(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 November 2002 (21.11.2002)

PCT

(10) International Publication Number
WO 02/092120 A1

(51) International Patent Classification[7]: A61K 38/04, 38/08, C07K 4/12, 7/02

(21) International Application Number: PCT/US02/15217

(22) International Filing Date: 14 May 2002 (14.05.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/290,646 15 May 2001 (15.05.2001) US

(71) Applicants *(for all designated States except US)*: **LUDWIG INSTITUTE FOR CANCER RESEARCH** [US/US]; 605 Third Avenue, New York, NY 10158 (US). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, rue Michel Ange, F-75794 Paris Cedex 16 (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GAIRIN, Jean, Edouard** [FR/FR]; Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR5089, 205, route de Robonne, F-31400 Toulouse (FR). **BLACHET, Jean-Sebastien** [FR/FR]; Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR5089, 205, route de Robonne, F-31400 Toulouse (FR). **DUFAU, Isabelle** [FR/FR]; Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR5089, 205, route de Robonne, F-31400 Toulouse (FR). **NGUYEN, Christophe** [FR/FR]; Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR5089, 205, route de Robonne, F-31400 Toulouse (FR). **MONSARRAT, Bernard** [FR/FR]; Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR5089, 205, route de Robonne, F-31400 Toulouse (FR). **VALMORI, Danila** [IT/CH]; CHUV, CH-1011 Lausanne (CH). **ROMERO, Pedro** [CO/CH]; University of Lausanne, CH-1066 Epalinges (CH). **CEROTTINI, Jean-Charles** [CH/CH]; University of Lausanne, CH-1066 Epalinges (CH).

(74) Agent: **SCHOFIELD, Mary, Anne**; Fulbright & Jaworski L.L.P., 801 Pennsylvania Avenue, N.W., Washington, DC 20004-2623 (US).

(81) Designated State *(national)*: US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/092120 A1

(54) Title: STRUCTURALLY MODIFIED PEPTIDES AND USES THEREOF

(57) Abstract: This invention relates to modified peptides that bind to an HLA molecule, particularly an HLA-A*0201 molecule wherein the modified peptides have a binding efficiency and immunogenicity similar to that of the parental non-modified peptide. This invention relates particularly to modified analogs of the tumor associated antigen Melan-A_{26-35}A27L that have at least, one or more modified amino acid at positions lor 2 or (b) one or more modified amino acid at positions 8, 9 or 10. The isolated modified peptides have a half-life in human serum of at least about 12 hours. Also disclosed are methods of using 10 such modified peptides. The invention also relates to methods for using the modified peptides of this invention.

STRUCTURALLY MODIFIED PEPTIDES AND USES THEREOF

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/290,646 filed May 15, 2001.

FIELD OF THE INVENTION

This invention relates to modified peptides that bind to MHC molecules and also promote an immunological response by cytolytic T cells (CTLs). The modified peptides are capable of activating CTLs that recognize the peptides in their unmodified form. In one embodiment the modified peptides display an enhanced resistance to proteases and an enhanced ability to stimulate CTLs as compared to the unmodified peptide. The modified peptides are useful in many ways, e.g., as immunogens and as materials which target and bind MHC/HLA molecules.

BACKGROUND AND PRIOR ART

The study of the recognition or lack of recognition of foreign antigen by a host organism has proceeded in many different directions. Understanding of the field presumes some understanding of both basic immunology, and protein chemistry.

Antigen recognition by the T-lymphocytes is restricted by cell-surface glycoproteins encoded by the highly polymorphic genes of the major histocompatibility complex (MHC) molecules. This discrimination requires a T cell specific for a particular combination of an MHC/HLA molecule and a peptide of the antigen rather than the intact foreign antigen itself. If a specific T cell is not present, there is no T cell response even if its partner MHC/peptide complex is present. Similarly, if the T cell is present but the specific MHC/peptide complex is absent, there is no T cell response.

Much work has focused on the mechanisms by which proteins are processed into the MHC/HLA binding peptide. See, in this regard, Cresswell, *Nature* 343: 593-594 (1990); Aichinger, et al. *Biochemical Society Trans*, 23: 657-659, (1995); Fremont et al., *Science* 257: 919 (1992); and Lanzavecchia, Science 260:937-943 (1993); Matsumura et al., *Science* 257: 927 (1992); Latron et al., *Science* 257: 964 (1992). Thus, specific T cell immunity is controlled by two selective and independent binding events: first, binding of the peptide fragments of the antigens by the MHC class molecules, and second, binding

of the resulting complexes by the clonotypic antigen receptors of the T cell. See, in this regard, Ada, *Immunology and Cell Biology* 72:447-454 (1994).

MHC class I molecules are expressed by almost all nucleated cells of the body and present peptides, usually 8 to 11 amino acids derived from intracellular proteins, to cytotoxic T cells expressing the CD8 co-receptor. See Roitt, et al. *Immunology* (Mosby-Year Book Europe, 1993). Activation of the cytotoxic T cell, in turn, results in the destruction of the target cells by apoptosis induced by perforin/granzyme and/or Fas ligand.

Tumor antigens are characteristic of tumor tissue and thus may be considered tissue specific. Tumor antigens result from alterations that frequently occur in malignant transformation of normal tissue. The alteration may be quantitative in that a particular normal antigen may decrease or increase in concentration, or the alteration may be qualitative in that a new antigen may appear. Normal antigens that have increased concentration in tumors are generally referred to as "Tumor Associated Antigens" or "TAAs". A new antigens, foreign to the host are termed "Tumor-Specific Antigens" or "TSAs" and may be present as new cell-surface structures or as new intracellular structures in the cytoplasm or nucleus.

Tumor specific antigens were first clearly demonstrated in mice that had been immunized with cells from a methylcholanthrene-induced sarcoma taken from syngeneic mice. These molecules were "recognized" by T cells in the recipient animal, and provoked a cytolytic T cell ("CTL" hereafter) response with lysis of the transplanted cells. The antigens expressed by the tumors and which elicited the T cell response were found to be different for each tumor. See Prehn, et al., *J. Natl. Canc. Inst.* 18: 769-778 (1957); Klein et al., *Cancer Res.* 20: 1561-1572 (1960); Gross, *Cancer Res.* 3: 326-333 (1943), Basombrio, *Cancer Res.* 30: 2458-2462 (1970) for general teachings on inducing tumors with chemical carcinogens and differences in cell surface antigens. This class of antigens has come to be known as "Tumor Specific Transplantation Antigens" or "TSTAs". Following the observation of the presentation of such antigens when induced by chemical carcinogens, similar results were obtained when tumors were induced *in vitro* via ultraviolet radiation. See Kripke, *J. Natl. Canc. Inst.* 53: 333-1336 (1974) Prehn, and Main, *Journal of Natl. Cancer Inst.* 18:769 (1974).

While T cell mediated immune responses were observed for the types of tumor described *supra*, spontaneous tumors were thought to be generally non-immunogenic. These were therefore believed not to present antigens that provoked a response to the tumor in the tumor carrying subject. See Hewitt, et al., *Brit. J. Cancer* 33: 241-259 (1976).

The family of tum$^-$ antigen presenting cell lines are immunogenic variants obtained by mutagenesis of mouse tumor cells or cell lines, as described by Boon et al., *J. Exp. Med.* 152: 1184-1193 (1980), the disclosure of which is incorporated by reference. To elaborate, tum$^-$ antigens are obtained by mutating tum + tumor cells. Tum+ cells do not generate an immune response in syngeneic mice and will form tumors (i.e., "tum+" cells). When these tum+ cells are mutagenized, they are rejected by syngeneic mice, and fail to form tumors (thus "tum$^-$"). See Boon et al., *Proc. Natl. Acad. Sci. USA* 74: 272 (1977), the disclosure of which is incorporated by reference. Many tumor types have been shown to exhibit this phenomenon. See, e.g., Frost et al., *Cancer Res.* 43: 125 (1983).

It appears that tum$^-$ variants fail to form progressive tumors because they elicit an immune rejection process. The evidence in favor of this hypothesis includes the ability of "tum$^-$" variants of tumor cells, i.e., those which do not normally form tumors, to do so in mice with immune systems suppressed by sublethal irradiation, Van Pel et al., *Proc. Natl, Acad. Sci.* USA 76: 5282-5285 (1979); and the observation that intraperitoneally injected tum$^-$ cells of mastocytoma P815 multiply exponentially for 12-15 days, and then are eliminated in only a few days in the midst of an influx of lymphocytes and macrophage (Uyttenhove et al., *J. Exp. Med.* 152: 1175-1183 (1980)). Further evidence includes the observation that mice acquire an immune memory which permits them to resist subsequent challenge to the same tum$^-$ variant, even when immunosuppressive amounts of radiation are administered with the following challenge of cells (Boon et al., *Proc. Natl, Acad. Sci. USA* 74: 272-275 (1977); Van Pel et al., *supra*; Uyttenhove et al., *supra*). Later research demonstrated that when spontaneous tumors were subjected to mutagenesis, immunogenic variants were produced which did generate a response. Indeed, these variants were able to elicit an immune protective response against the original tumor. See Van Pel et al., *J. Exp. Med.* 157: 1992-2001 (1983). Thus, it has

been shown that it is possible to elicit presentation of a so-called "Tumor Rejection Antigen" or"TRA" in a tumor which is a target for a syngeneic rejection response. Similar results have been obtained when foreign genes have been transfected into spontaneous tumors. See Fearson et al., *Cancer Res.* 48: 2975-1980 (1988).

TRAs may or may not induce antibodies and have been studied by characterizing the response of cytolytic T cell *in vitro*, i.e., by analyzing the recognition of the TRA by a particular cytolytic T cell subset. The subset proliferates upon recognition of the presented TRA, and the cells presenting the TRA are lysed. Characterization studies have identified CTL clones which specifically lyse cells expressing the antigens. Examples of this work may be found in Levy et al., *Adv. Cancer Res.* 24: 1-59 (1977); Boon et al., *J. Exp. Med.* 152: 1184-1193 (1980); Brunner et al., *J. Immunol.* 124: 1627-1634 (1980); Maryanski et al., *Eur. J. Immunol.* 124: 1627-1634 (1980); Maryanski et al., *Eur. J. Immunol.* 12: 406-412 (1982); Palladino et al., *Canc. Res.* 47: 5074-5079 (1987).

A tumor exemplary of the subject matter described *supra* is known as P815. See DePlaen et al., *Proc. Natl. Acad. Sci. USA* 85: 2274-2278 (1988); Szikora et al., *EMBO J* 9: 1041-1050 (1990), and Sibille et al., *J. Exp. Med.* 172: 35-45 (1990), the disclosures of which are incorporated by reference. The P815 tumor is a mastocytoma, induced in a DBA/2 mouse with methylcholanthrene and cultured as both an in vitro tumor and a cell line. The P815 line has generated many tum$^-$ variants following mutagenesis, including variants referred to as P91A (DePlaen, *supra*), 35B (Szikora, *supra*), and P198 (Sibille, *supra*). In contrast to TRAs, the tum$^-$ antigens are only present after tumor cells are mutagenized. TRAs are present on tumor cells without mutagenesis.

Thus, a cell line can be tum+, such as the line referred to as "P1", and can be provoked to produce tum$^-$ variants. Since the tum$^-$ phenotype differs from that of the parent cell line, one expects a difference in the DNA of tum$^-$ cell lines as compared to their tum+ parental lines, and this difference can be exploited to locate the gene of interest in tum$^-$ cells. As a result, it was found that genes of tum$^-$ variants such as P91A, 35B and P198 differ from their normal alleles by point mutations in the coding regions of the gene. See Szikora and Sibille, *supra*, and Lurquin et al., *Cell* 58: 293-303 (1989). These papers also demonstrated that peptide derived from the tum$^-$ antigen are presented by the H-2 molecule for recognition by CTLs. P91A is presented by Ld, P35 by Dd and P198 by Kd.

Human melanoma cells also bear antigens that are recognized by autologous CD8+ cytolytic T cells, which can be derived from blood lymphocytes or from tumor-infiltrating lymphocytes. PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, discloses the MAGE gene family. The MAGE family is a family of genes encoding tumor rejection antigen precursors, TRAPs, which are proteins that are processed into TRAs. The TRAs are expressed on cell surfaces and lead to lysis of the tumor cells by specific cytolytic T lymphocytes. See Traversari et al., *Immunogenetics* 35: 145 (1992); van der Bruggen et al., *Science* 254: 1643 (1991), for further information on this family of genes. Also, see U.S. Patent No. 5,342,774,which discloses the "MAGE" family of tumor rejection antigen precursors, and U.S. Patent No. 5,405,940, PCT/US93/07421; Falk et al., *Nature* 351: 290-296 (1991); Engelhard, *Ann Rev. Immunol.* 12: 181-207 (1994); Ruppert et al., *Cell* 74: 929-937 (1993); Rötzschke et al., *Nature* 348: 252-254 (1990); Bjorkman et al., *Nature* 329: 512-518 (1987); Traversari et al., *J. Exp. Med.* 176: 1453-1457 (1992) all incorporated by reference. These references teach that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind to least one HLA molecule.

A cursory review of the development of the field may be found in Barinaga, "Getting Some 'Backbone': How MHC Binds Peptide", *Science* 257: 880 (1992); also, see Fremont et al., *Science* 257: 919 (1992); Matsumura et al., *Science* 257: 927 (1992); Latron et al., *Science* 257: 964 (1992). These papers generally point to a preference that the peptide which binds to an MHC/HLA molecule be nine amino acids long (a "nonapeptide"), and to the importance of two so-called MHC anchor residues (most commonly, the second or third and ninth residues of the nonapeptide).

A survey of the relevant literature (*supra*) demonstrates that various peptides, usually eight, nine, or ten amino acids in length, complex with MHC molecules and present targets for recognition by cytolytic T cells. A great deal of study has been carried out on melanoma, and melanoma antigens that are recognized by cytolytic T cells are now divided into three broad categories. The first, which includes many of the antigens discussed *supra* (e.g., MAGE), are expressed in some melanomas, as well as other tumor types, and normal testis and placenta. The antigens are the expression product of normal genes which are usually silent in normal tissues.

A second family of melanoma antigens includes antigens which are derived from mutant forms of normal proteins. Examples of this family are MUM-1 (Coulie, et al., *Proc. Natl. Acad. Sci. USA* 92:7976-7980 (1955)); CDK4 (Wölfel, et al., *Science* 269:1281-1284(1955)); β-catenin (Robbins, et al., *J. Exp. Med.* 183:1185-1192 (1996)); and HLA-A2 (Brandel, et al., *J. Exp. Med.* 183:2501-2508 (1996)).

A third category, also discussed, *supra*, includes the differentiation antigens which are expressed by both melanoma and melanocytes. Exemplary are tyrosinase gp100, gp75, and Melan A/Mart-1. See U.S. Patent No. 5,620,886 incorporated by reference, with respect to Melan-A. See Wölfel, et al., *Eur. J. Immunol.* 24: 759 (1994) and Brichard, et al., *Eur. J. Immunol.* 26: 224 (1996) for tyrosinase; Kang, et al., *J. Immunol.* 155: 1343 (1995); Cox, et al., Science 264: 716 (1994); Kawakami, et al., *J. Immunol.* 154: 3961 (1995) for gp 100; Wang, et al., *J. Exp. Med.* 183: 1131 (1996) for gp 75 (all incorporated herein by reference).

CTLs have been identified in peripheral blood lymphocytes and tumor infiltrating lymphocytes of melanoma patients who are HLA-A*0201 positive. See Kawakami, et al., *Proc. Natl. Acad. Sci. USA* 91:3515 (1994); Coulie, et al., *J. Exp. Med.* 180:35 (1994). When ten HLA-A*0201 restricted Melan-A specific CTLs were derived from different patients were tested, nine of them were found to recognize and react and the peptide Ala Ala Gly Ile Gly Ile Leu Thr Val, (SEQ ID NO: 1), which consists of amino acids $_{27\text{-}35}$ of Melan-A. (Kawakami, et al., *J. Exp. Med* 180:347-352 (1994)). Rivoltini, et al., *J. Immunol* 154:2257 (1995), showed that Melan-A specific CTLs could be induced by stimulating PBLs from HLA-A*0201 positive normal donors, and melanoma patients, using SEQ ID NO: 1. The strength of this response has led to SEQ ID NO: 1 being proposed as a target for vaccine development. It has now been found, however, that a decapeptide, i.e.,

Glu Ala Ala Gly Ile Gly Ile Leu Thr Val (SEQ ID NO: 2),

is actually a better target than SEQ ID NO: 1. This recognition has led to work set forth herein, which is part of the invention.

The majority of peptides which have been identified as binding to HLA-A*0201 are 9 or 10 amino acids in length, and are characterized by two anchor residues. The first is Leu or Met at position 2, and the second is Leu or Val at position 9. See Falk, et al.,

Nature 351:290 (1991). Ruppert, et al., *Cell* 74:929 (1993), show that amino acids found at other positions within a nona-or decapeptide may also have a role in the peptide -HLA-A*0201 interaction. They demonstrate, e.g., that a negatively charged residue or proline at position 1 was associated with poor HLA-A*0201 binding.

What is interesting about this work is that the two peptides represented by SEQ ID NOS: 1 and 2 do not possess the major anchor residue at position 2 and, the strong binder SEQ ID NO: 2 has a negatively charged residue at position 1.

A strong binder is not necessarily a stable binder, meaning that the interaction between peptide and HLA molecule may be, and is, brief. When it is desired to induce CTLs, to identify them or to carry out other types of experiments, it would be desirable to have a peptide with the ability to bind to an MHC Class I molecule with high affinity and form stable complexes.

The characterization of tumor associated antigens recognized by CD8+ cytotoxic T lymphocytes (CTL) provided the basis for cancer immunotherapy trials. These small peptides (8 to 11 amino acids) are presented to CTLs on the surface of tumor cells in complex with MHC class I molecules. Several groups of tumor associated antigens have been identified thus far and peptides derived from these antigens are currently assayed in cancer immunotherapy clinical trials.

Melan-A/MART-1 (MelanA thereafter) is a member of the differentiation tumor associated antigens group. It is expressed in melanomas as well as in normal melanocytes but not in other normal cells. Melan-A_{27-35} (AAGIGILTV, SEQ ID NO: 1) or Melan-A_{26-35} epitopes (EAAGIGILTV, SEQ ID NO: 2) are recognized by specific CTL in an HLA-A*0201 restricted fashion. Tumoricidal CD8+ T lymphocytes specific for the Melan-A_{26-35} peptide are found in tumor infiltrated lymph nodes (TILN) and tumor infiltrating lymphocytes (TIL) from melanoma patients. A mutated analog of Melan-A antigen, peptide Melan-A_{26-35}A27L (Glu Leu Ala Gly Ile Gly Ile Ley Thr Val, ELAGIGILTV, SEQ ID NO: 3) described by Valmori et al. has enhanced MHC binding and immunogenicity (Valmori et al., *J. Immunol.* 1998, 160 : 1750-1758, Men et al., *J. Immunol.* 1999, 162 : 3566-3573, incorporated herein by reference). Both Melan-A_{26-35} and Melan-A_{26-35}A27L are currently being assayed in melanoma immunotherapy clinical trials. Use of peptides in specific immunotherapy offers a number of advantages,

especially a specific response and a low toxicity. However, a severe limitation to the use of peptides as efficient therapeutic agents is their high sensitivity to proteolytic degradation in biological fluids or tissues.

Described herein are modified analogs of the tumor antigen Melan-A_{26-35} A27L that are resistant to peptidase degradation and retain the biological properties of the parental peptide.

SUMMARY OF THE INVENTION

This invention relates to a fine analysis of the molecular proteolytic degradation mechanism of a tumor associate antigen particularly a Melan-A peptide, Melan A_{26-35} A27L peptide, in human serum. This invention also relates to structurally modified analogs of tumor associated antigens, particularly Melan A/Mart-1 peptides.

The modified peptides of this invention are resistant to proteolytic degradation and yet do not display a loss or substantial reduction in the immunological properties displayed by the corresponding parental peptide. Described herein is the synthesis of the modified peptides and an analysis of their resistance to proteolysis, their capacity to be presented by MHC molecules (measurement of MHC binding affinity), particularly an HLA-A*0201, their antigenicity (ability to provoke lysis of target cells by CTLs which recognize the parental peptide in complex with the MHC molecule, e.g., the ability to provoke lysis of target cells by Melan-A_{26-35} A27L-specific cytolytic T lymphocytes) and their immunogenicity (ability to generate specific CTL capable of recognizing the non-modified parental peptide and tumor cells that naturally express the tumor associated antigen, e.g., a Melan-A antigen) as compared to that of the parental peptide.

The structurally modified peptides of this invention are resistant to proteolysis, particularly proteolysis by amino- and dipeptidyl-carboxy-peptidase (ACE : angiotensin-converting enzyme sub-family). Preferably the modified peptides of this invention display a minimal reduction in the immunological properties of the peptide in its non-modified form. Preferably the modified peptide is a modified Melan A peptide, e.g., a modified Melan A_{27-35} or a modified Melan A_{26-35} peptide. More preferably the modified peptide is a modified Melan-A_{26-35} A27L peptide. Resistance to proteolysis may be

manifested as an extended half life in human serum. Preferably the peptides have a half life in human serum of at least 12 hours. More preferably the peptides of this invention have a half life of at least about 24 hours.

In one embodiment of this invention the peptides are mono-substituted at one of amino acid positions 1, 2, 8, 9 or 10, preferably at the amino acid position 1 or 2. In another embodiment of this invention the peptides of this invention comprise two or more modified amino acids at positions 1, 2, 8, 9 or 10. The peptides may be modified at (a) at least one of position 1 or 2 and (b) at least one of positions 8, 9 or 10.

The modification of the peptide is preferably one that renders the peptide resistant to proteolysis in serum, particularly human serum, as compared to the non-modified peptide. The modified peptide comprises an amino acid modified at the alpha carboxy, the alpha amino or the peptide bond at amino acid positions 1, 2, 8, 9 or 10, e.g., the amino acid a β-amino acid, e.g., β-alanine or β-glutamic acid, a D-amino acid (d-a.a.), or a cyclic amino acid, e.g., a pyro-glutamic a.a., or an amino acid with a methylation of an α carboxy (αME-peptide), methylation of a nitrogen engaged in peptidic bond formation (NMe-peptide), acetylation of a terminal nitrogen (acetyl peptide), amidation of a terminal carboxylic group (amide-peptide), hydroxylation of a terminal nitrogen (NOH-peptide). The modified peptide may also comprise one or more non-peptide bonds, e.g., a reduced peptide bond ($\Psi_{1\text{-}2}(CH_2\text{-}NH)$ or a retro-inverso peptide bond ($\Psi_{1\text{-}2}(CO\text{-}NH_2)$). Preferably the amino acid is one having αMe modification . The modified peptide may substitute an amino acid at position 1, 2, 8, 9 or 10 in the parental peptide.

Preferably the peptides of this invention comprise a modification of an amino acid at position 1, 2, 8, 9 or 10, e.g., both one or more modified amino acid at position one or two, and one or more modified amino acid at position 8, 9 or 10. The peptides of this invention may comprise a modification of a peptide bond between amino acids 1 and 2 or between amino acids at positions 8 and 9 or 9 and 10. Preferably the peptide bond modified is the bond between amino acids at position 1 and 2 or positions 8 and 9. Preferably the modification is a methylation of an α carboxy (αME-peptide) methylation of a nitrogen engaged in peptidic bond formation (NMe-peptide), acetylation of a terminal nitrogen (acetyl peptide), amidation of a terminal carboxylic group (amide-peptide), reduced bond ($\Psi_{1\text{-}2}(CH_2\text{-}NH)$, β-amino acid (βaa), e.g., β-alanine, β-glutamic acid, D-amino acid (d-aa), hydroxylation of a terminal nitrogen (NOH-peptide), retro-

inverso peptide bond ($\Psi_{1\text{-}2}(CO\text{-}NH_2)$), and cyclic amino acid, e.g., pyro-glutamic a.a.. Preferably the peptides of this invention containing modified amino acids, particularly at one of positions 1, 2 8, 9 or 10 display very similar HLA binding and CTL recognition compared to the non-modified parental peptide. Preferably the HLA is an HLA-A *0201 molecule. Preferably the peptides of this invention consist of an amino acid sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43

Immunogenicity may be assayed by a variety of methods routinely used by one of skill in the art. For example, the immunogenicity of the peptides may be assayed *in vitro* by their ability to stimulate peripheral blood lymphocytes. In this case the peripheral blood lymphocytes are preferably from healthy HLA-A*0201+ donors. The level of stimulation may be determined using any number of well-known assays, e.g., by flow cytometry analysis wherein the peptides are assayed for their ability to induce an increase in the number of cells specific for the modified or non-modified parental peptide. T cells specific for the modified peptides of this invention may also be assayed *in vitro* for expansion of their cytolytic activity. Preferably the CTLs induced with the modified peptides of this invention are able to recognize the non-modified parental peptide, e.g. Melan $A_{26\text{-}35}$ A27L, and lyse tumor cell line that present the natural peptide, e.g., Melan $A_{26\text{-}35}$.

Another embodiment of this invention are CTLs which recognize the modified peptides and also recognize the non-modified parental peptide. Preferably the CTLs recognized the modified Melan A peptides of this invention, the parental Melan A peptide, and the natural Melan A peptide. More preferably the CTLs recognize the modified peptides of this invention, in addition to Melan $A_{26\text{-}35}$ A27L and Melan $A_{26\text{-}35}$.

A further embodiment of this invention is a composition comprising the peptides of this invention and a pharmaceutically acceptable carrier. Any pharmaceutical carrier well known in the art is suitable. Preferably the peptide is a modified Melan A peptide, more preferably a Melan $A_{26\text{-}35}$ or Melan $A_{27\text{-}35}$ peptide and most preferably a modified Melan $A_{26\text{-}35}$ A27L peptide

Still another embodiment of this invention is a method of stimulating a response from a CTL specific for a complex of Melan A and an MHC molecule, particularly an HLA-A 0*0201 molecule. The methods comprise contacting a sample of cells containing CTL precursors with a peptide of this invention, wherein the amount of the peptide is sufficient to form a complex with an MHC molecule expressed on the surface of a cell, and provoke a response by CTLs specific for the complex of peptide and the MHC molecule. The stimulated CTL cells lyse cells presenting a complex of the peptide and the MHC molecule. Preferably the peptide is a modified Melan A_{26-35} A27L peptide wherein at least one of amino acids at positions 1 or 2 is modified. Preferably the peptide comprises one or more modified amino acids at position 1 or 2 and one or more modified amino acids at positions 8, 9, or 10. Preferably an amino acid at position 1 or 2 and an amino acid at position 8, 9 or 10 is modified or the peptide bond between amino acids 8 and 9 is modified.

Also a part of the invention are isolated cytolytic T cell lines which are specific for complexes of these peptides and their MHC binding partner, i.e., an HLA molecule, such as an HLA-A2 molecule, HLA-A*0201 being especially preferred.

The ability of these peptides to bind to HLA molecules makes them useful as agents for determining presence of HLA-A2 positive cells, such as HLA-A*0201 positive cells, by determining whether or not the peptides bind to cells in a sample. This "ligand/receptor" type of reaction is well known in the art, and various methodologies are available for determining it.

The peptides may be combined with peptides from other tumor rejection antigens to form 'polytopes'. Exemplary peptides include those listed in U.S. Patent Application Serial Numbers 08/672,351, 08/718,964, now U.S. Patent No. 5,932,694 , 08/487,135 now U.S. Patent No. 5,821,122, 08/530,569 and 08/880,963, now U.S. Patent No. 6,025,470, 08/795,733, now U.S. Patent No. 6,087,441, all of which are incorporated by reference.

Additional peptides which can be used are those described in the following references, all of which are incorporated by reference: U.S. Patent Nos. 5,405,940; 5,487,974; 5,519,117; 5,530,096; 5,554,506; 5,554,724; 5,558,995; 5,585,461; 5,589,334; 5,648,226; and 5,683,886; PCT International Publication Nos. 92/20356; 94/14459;

96/10577; 96/21673; 97/10837; 97/26535; 97/31017 and 98/12879, as well as pending U.S. Application Serial No. 08/713,354. Most of the modification of tumor rejection antigens and tumor associated antigens reported prior to this invention result in a reduction or a complete loss of peptide binding to HLA-A*0201

Polytopes are groups of two or more potentially immunogenic or immune stimulating peptides, which can be joined together in various ways, to determine if this type of molecule will stimulate and/or provoke an immune response. These peptides can be joined together directly, or via the use of flanking sequences. See Thompson et al., *Proc. Natl. Acad. Sci. USA* 92(13): 5845-5849 (1995), teaching the direct linkage of relevant epitopic sequences. The use of polytopes as vaccines is well known. See, e.g. Gilbert et al., *Nat. Biotechnol.* 15(12): 1280-1284 (1997); Thomson et al., supra; Thomson et al., *J. Immunol.* 157(2): 822-826 (1996); Tam et al., *J. Exp. Med.* 171(1): 299-306 (1990), all of which are incorporated by reference. The Tam reference in particular shows that polytopes, when used in a mouse model, are useful in generating both antibody and protective immunity. Further, the reference shows that the polytopes, when digested, yield peptides which can be and are presented by MHCs. Tam shows this by showing recognition of individual epitopes processed from polytope 'strings' via CTLs. This approach can be used, e.g., in determining how many epitopes can be joined in a polytope and still provoke recognition and also to determine the efficacy of different combinations of epitopes. Different combinations may be 'tailor-made' for the patients expressing particular subsets of tumor rejection antigens. These polytopes can be introduced as polypeptide structures, or via the use of nucleic acid delivery systems. To elaborate, the art has many different ways available to introduce DNA encoding an individual epitope, or a polytope such as is discussed supra. See, e.g., Allsopp et al., *Eur. J. Immunol.* 26(8); 1951-1959 (1996), incorporated by reference. Adenovirus, pox-virus, Ty-virus like particles, plasmids, bacteria, etc., can be used. One can test these systems in mouse models to determine which system seems most appropriate for a given, parallel situation in humans. They can also be tested in human clinical trials.

Also a feature of the invention is the use of these peptides to determine the presence of cytolytic T cells in a sample. It was shown, supra, that CTLs in a sample will react with peptide/MHC complexes. Hence, if one knows that CTLs are in a sample, HLA-A2 positive cells can be "lysed" by adding the peptides of the invention to HLA-A2

positive cells, such as HLA-A*0201 positive cells, and then determining, e.g., radioactive chromium release, TNF production, etc. or any other of the methods by which T cell activity is determined. Similarly, one can determine whether or not specific tumor infiltrating lymphocytes ("TILs") are present in a sample, by adding one of the claimed peptides with HLA-A2 positive cells to a sample, and determining lysis of the HLA-A2 positive cells via, e.g., 51Cr release, TNF presence and so forth. In addition, CTL may be detected by ELISPOT analysis. See for example Schmittel et al (1997). *J. Immunol.* Methods 210: 167-174 and Lalvani et al (1997). *J. Exp. Med.* 126: 859 or by FACS analysis of fluorogenic tetramer complexes of MHC Class I/peptide (Dunbar et al (1998), *Currrent Biology* 8: 413-416. All are incorporated by reference.

Of course, the peptides may also be used to provoke production of CTLs. CTL precursors develop into CTLs when confronted with appropriate complexes. By causing such a "confrontation" as it were, one may generate CTLs. This is useful in an in vivo context, as well as ex vivo, for generating such CTLs.

Also a feature of the invention are compositions which comprise at least one of the peptides of the invention, in combination with at least one additional agent, e.g., an adjuvant. Such compositions can be used, e.g., to generate immune responses, preferably in humans, as part of a therapeutic regime, but also in subject non-human animals, to generate immune components which can then be used to treat humans, or diagnostically. The artisan of ordinary skill is familiar with such adjuvants, and thus these do not have to be set forth here.

These compositions can also include so-called co-stimulatory molecules. These are molecules which are proteins, or encode proteins, that interact with molecules on the surface of T cells, thereby co-stimulating a T cell already stimulated by formation of an MHC molecule/antigen/T cell receptor interaction. Such co-stimulatory molecules enhance antitumor immunity, and CTL proliferation. Exemplary of such co-stimulatory molecules are those known as "B7-1" and "B7-12," or CD80 and CD86, respectively. See Zhang, et al., *Proc. Natl. Acad. Sci. USA* 95(11):6284-6289 (1998), incorporated by reference.

Such co-stimulatory molecules can be combined with, e.g. interleukins, such as IL-6 and IL-12. See Gajewski, et al., *J. Immunol* 154:5637-5648 (1995). As noted,

supra, the co-stimulatory molecules may be administered in the form of a nucleic acid molecule. Such an approach can be useful in connection with CTL expansion for adoptive transfer immunotherapy (Wang et al., *J. Immunother. Emphasis Tumor Immunol.* 19:1-8 (1996)). The requisite nucleic acid molecules can be administered in the form of "naked" DNA (Kim et al., *Nat. Biotechnol* 15(7):641-646 (1997)), as well as in the form of recombinant vectors, such as adenovirus and pox virus vectors. See Wendtner et al., *Gene Ther.* 4(7):726-735 (1997). All of these systems can be adapted so that the co-stimulatory molecule is expressed together with other molecules of choice, including the peptides, adjuvant molecules, and so forth.

Other features of the invention will be clear to the skilled artisan, and need not be repeated here.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the recognition of the Melan-A_{26-35} A27L modified analogs by specific CTL raised against Melan-A_{26-35} A27L. The symbols used are: ▲ Melan-A_{27-35} (AAGIGILTV, SEQ ID NO: 1), ● Melan-A_{26-35} (EAAGIGILTV, SEQ ID NO: 2), ■ Melan-A_{26-35} A27L (ELAGIGILTV, SEQ ID NO: 3), ◊ [αMeL2, V10-amide]-Melan-A_{26-35} A27L (SEQ ID NO:14), ○[αMeL2, αMeL8]-Melan-A_{26-35} A27L (SEQ ID NO:13), Δ [βA1, Ψ_{8-9} (CH2-NH)]-Melan-A_{26-35} A27L (SEQ ID NO: 17), □ [αMeL2, NMeT9]-Melan-A_{26-35} A27L (SEQ ID NO: 15), x [αMeL2, dT9]-Melan-A_{26-35} A27L (SEQ ID NO: 16).

Figure 2 depicts the mmunogenicity of the Melan-A_{26-35} A27L modified analogs. * : Melan-A designation correspond to modified peptide of Melan-A_{26-35} A27L antigen.

Figure 3 depicts the recognition of tumor cells by CTL generated with the Melan-A_{26-35} A27L non-natural modified analogs.

Figure 4 depicts the recognition of Melan-A_{26-35} by the CTL generated with the Melan-A_{26-35} A27L modified analogs.

Figure 5 depicts the recognition of tumor cells by CTL generated with the Melan-A_{26-35} A27L modified analogs.

Figure 6 depicts the antigenic specificity and tumor recognition of CTL induced with the modified Melan-A_{26-35} A27L analogs. Fig. 6A is a dot plot representation of cytometry analysis of culture after in vitro stimulation with Melan-A_{26-35} A27L. Fig 6 B1-B8, depicts Melan-A antigenic peptide recognition by the modified Melan-A analogs-specific CTL. Fig. 6 C1-C8 depicts tumor cell line recognition by the modified Melan-A analogs specific CTL. The Melan-A analog used for *in vitro* stimulation is indicated before the B and C series of figures.

DETAILED DESCRIPTION OF THE INVENTION

Use of peptides in specific immunotherapy offers a number of advantages, especially a specific response and a low toxicity. However, a severe limitation to the use of peptides as efficient therapeutic agents is their high sensitivity to proteolytic degradation in biological fluids or tissues.

Disclosed herein is an approach for the design of analogs of the tumor antigens, particularly modified analogs of Melan-A_{26-35} A27L, that are resistant to peptidase degradation and still retain the biological properties of the parental peptide, e.g., Melan-A_{26-35} A27L. The first step consisted in the fine analysis of the molecular proteolytic degradation mechanism of Melan-A_{26-35} A27L in human serum. The second step was the synthesis of the structurally modified analogs based on the knowledge of the degradation process of Melan-A and evaluation of their efficient resistance to peptidase activities. In the third step, their capacity to be presented by HLA-A*0201 (measurement of MHC binding affinity), their antigenicity (ability to sensitize target cells to lysis by Melan-A_{26-35} A27L specific cytolytic T lymphocytes) and their immunogenicity (ability to generate specific CTL capable to recognize parental peptide and tumor cells that naturally express Melan-A antigens) were analyzed and compared to that of the parental antigen Melan-A_{26-35} A27L.

Disclosed herein are non-natural modified Melan-A peptides [αMeL2, αMeL8]-Melan-A and [αMeL2, amide]-Melan-A which are fully resistant to degradation by proteases and retain the potent immunogenicity of the parental peptide, Melan A_{26-35} A27L. Specific cytolytic T lymphocytes (CTL) induced by the modified Melan-A peptides [αMeL2, αMeL8]-Melan-A and [αMeL2, amide]-Melan-A were able to recognize efficiently the parental peptide and tumor cell lines that naturally express the

Melan-A antigen. Some of the structural modifications decreased biological properties of the antigen. These peptides [αMeL2, αMeL8]-Melan-A and [αMeL2, amide]-Melan-A displayed a reduced binding to HLA-A*0201 and a reduced recognition by the Melan-A_{26-35} A27L-specific CTL as compared to the parental antigen. These results have important implications in peptide-based immunotherapy of melanoma.

Additional structural modifications of Melan-A_{26-35} A27L antigenic peptide resulted in the generation of modified peptides with minimal decrease of the immunological properties of the antigen. Mono-protected Melan-A_{26-35} A27L analogs were synthesized having structural modifications such as, e.g., βa.a., N-hydroxy-a.a., retro-inverso peptide bond, pyro-glutamic a.a.. These modified peptides were assayed for their HLA-A*0201 binding properties and their recognition by Melan-A_{26-35} A27L specific CTL. The modifications that displayed the least reduction in HLA-A*0201 binding and CTL recognition were combined and doubly protected analogs which were fully resistant to proteolysis were generated. Several doubly-protected analogs displayed very similar HLA-A*0201 binding and CTL recognition compared to the parental peptide. Immunogenicity of these modified analogs of Melan-A_{26-35} A27L was assayed by *in vitro* stimulation of peripheral blood lymphocytes from healthy HLA-A*0201$^+$ donors. As shown by flow cytometry analysis using Melan-A-tetramers, three modified peptides were capable of inducing increased numbers of Melan-A specific cells as compared to the parental peptide. These results were observed in independent experiments with three different donors. After A2/Melan-A-tetramer guided cell sorting of the modified Melan-A specific T cells and their *in vitro* expansion the CTLs were assayed for their cytolytic activity. The CTL induced with the modified Melan-A analogs not only recognized cells presenting the modified Melan A_{26-35} A27L analog but also recognized the parental peptide and lysed tumor cell lines that naturally express Melan-A antigens.

Seven non natural modified Melan-A_{26-35} A27L analogs are fully protected against proteolytic degradation and are able to induce Melan-A specific anti-tumor CTLs. Three of the seven Melan-A analogs appear to be even more immunogenic than the parental Melan-A peptide.

EXAMPLES

EXAMPLE 1

A. Cells.

Tumor cell line Me 290 (HLA-A*0201+, Melan-A+) and Me 275 (HLA-A*0201+, Melan-A+) were established at the Ludwig Institute for Cancer Research, Lausanne Branch, from surgically excised melanoma metastasis from patient LAU203 and LAU50 respectively, and maintained in culture as previously described (Valmori et al., *J. Immunol.*, 160 : 1750-1758 (1998) incorporated herein by reference). The melanoma cell line NA8 do not express Melan-A gene (HLA-A*0201+, Melan-A-). T2 cells is an HLA-A*0201+ human T/B-cell hybrid which are peptide transporter deficient were cultured in DMEM 10% FCS supplemented with 0.55mM Arg, 0.24mM Asn and 1.5mM Gln. Melan-A specific CTL used for the recognition studies were a polyclonal monospecific line derived form patient LAU203 after in vitro stimulation of PBL with the peptide Melan-A_{26-35} A27L. The tyrosinase specific CTL clone 156/34 was derived after in vitro stimulation of TIL from patient LAU 156 with the natural tyrosinase$_{368-376}$ peptide (Tyr Met Asp Gly Thr Met Ser Gln Val, YMDGTMSQV, SEQ ID NO: 46).

B. Peptide synthesis.

Peptides were manually synthesized by the solid-phase method on an using the Fmoc chemistry and DIPC/HOAt (N, N diispropylcarbodiimmide / N-hydroxyaza-benzotiazole) coupling procedure. Peptide with modifications like β-a.a. (β-amino acid), d-a.a., αMe-a.a. or NMe-a.a. (see Table I and II) were synthesized by incorporation of the corresponding modified amino acid commercially available (Bachem AG, Budendorf, Switzerland (NMe-a.a.), Fluka Chemie GmbH, Bush, Switzerland (β-a.a.), Acros Organic France, Noisy-Le-Grand, France (αMe-a.a.)). αMe amino acid were sold in a racemic form. As a consequence, peptide with αMe modification were also obtain in a racemic form. Pure isomeric forms were isolated by RP-HPLC and named p1 and p2 (Table 1A).

The reduced bond $\Psi(CH_2\text{-}NH)$ (see Table IA and IIA) was formed by the reductive alkylation of a free amino group with a Fmoc protected amino aldehyde preformed according to the method developed by Fehrentz and Castro (Fehrentz and Castro, 1983, Synthesis, 676-678). Peptides were purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a C8 column (Aquapore (Brownlee)). Peptides

were used at a purity > 98%. Identity of the purified peptides was confirmed by mass spectrometry using electrospray ionisation (ESI-MS). Peptide stock solutions were made at a peptide concentration of 10-3 M in 100 % dimethylsulfoxyde (DMSO), and stored at -20°C.

N-terminal-hydroxy peptides were synthesized according to a previous study (Bianco et al., *J. Peptide Sci.*, 4 : 471-478 (1998), incorporated herein by reference). The retro-inverso bond Ψ(NH-CO) (see Table IB) was obtained by replacement of two sequential amino acids with a (R, S)-2-substituted malonate derivative and a gem-diaminoalkyl residue. Thus, [Ψ_{1-2}(NH-CO)]-Melan-A_{26-35} A27L modified peptide was synthesized using gem-diaminoalkyl residue corresponding to glutamic acid side chain and 2-substituted malonic acid corresponding to leucine side chain (2(R, S)-isobutylmalonic acid) (Guichard et al., *J. Med. Chem.*, 1996, 39 : 2030-2039, Dürr et al., *Angew. Chem. Int. Ed. Engl.*, 31(6) : 785-787 (1992)). Two diastereoisomers of the modified peptide were obtained and were isolated in pure isomeric form by RP-HPLC and named p1(3) and p2(4). Peptides were purified by RP-HPLC. Peptides were used at a purity > 98%. Identity of the purified peptides was confirmed by mass spectrometry using electrospray ionization (ESI-MS). Peptide stock solutions were made at a peptide concentration of 10^{-3} M in 100 % dimethylsulfoxyde (DMSO), and stored at -20°C.

Table IA. *Sequence, HLA-A*0201 binding properties and Melan-A specific CTL recognition of the non-natural Melan-A_{26-35} A27L analogues.*

Peptide name	Peptide sequence	HLA-A*0201 binding Competitor activity [a] IC_{50} [nM]	CTL recognition Antigenic activity CE_{50} [nM]
Melan-A_{27-35}	AAGIGILTV	50	4
Melan-A_{26-35}	EAAGIGILTV	20	0.25
Melan-A_{26-35} A27L	ELAGIGILTV	2	0.01
Flu MA	GILGFVFTL	< 0.01	-
Amino-protected analogs			
[βA1]-Melan-A [c]	[βA]-LAGIGILTV	0.5	0.01
[NMeL2]-Melan-A	E-[NMeL]-AGIGILTV	> 1000	30
[$\psi_{1-2}(CH_2\text{-}NH)$]-Melan-A	E-[$\psi(CH_2\text{-}NH)$]-LAGIGILTV	> 1000	50
Carboxy-protected analogs			
[αMeL8]-Melan-A	ELAGIGI-[αMeL]-TV	4	p_1 0.01 p_2 3
[NMeT9]-Melan-A	ELAGIGIL-[NMeT]-V	100	0.5
[dT9]- Melan-A	ELAGIGIL-[dT]-V	> 1000	4
[V10-amide]-Melan-A	ELAGIGILTV-[$CONH_2$]	20	0.02
[$\psi_{8-9}(CH_2\text{-}NH)$]-Melan-A	ELAGIGIL-[$\psi(CH_2\text{-}NH)$]-TV	300	0.3

Table IA Cont'

Peptide name	Peptide sequence	HLA-A*0201 binding Competitor activity [a] IC_{50} [nM]	CTL recognition Antigenic activity CE_{50} [nM]
Double-protected analogs			
[αMeL2, αMeL8]-Melan-A	E-[αMe]-LAGIGI-[αMeL]-TV	9	0.18
[αMeL2, V10-amide]-Melan-A	E-[αMe]-LAGIGILTV-[$CONH_2$]	p_1 >1000 p_2 100 [b]	p_1 2.5 p_2 0.1
[αMeL2, NMeT9]-Melan-A	E-[αMe]-LAGIGIL-[NMeT]-V	500	0.5
[αMeL2, dT9]-Melan-A	E-[αMe]-LAGIGIL-[dT]-V	90	10.5
[βA1, $\psi_{8-9}(CH_2\text{-}NH)$]-Melan-A	βALAGIGIL-[$\psi(CH_2\text{-}NH)$]-TV	20	0.35

[a] Competitor activity was measured on the basis of the inhibition of recognition of the $tyrosinase_{368-376}$ (YMDGTMSQV, SEQ ID NO: 46) antigenic peptide in the context of HLA-A*0201 by the tyrosinase peptide-specific CTL clone 156/34.

[b] In the case of α-methylated modified peptide, the two enantiomers separated by HPLC are indicated as p_1 and p_2 see "Material and Methods" peptide synthesis part for more details.

[c] Melan-A designation correspond to Melan-A_{26-35} A27L.

SEQ ID Nos (from top to bottom) = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17

Table IB. *Sequences, HLA-A*0201 binding properties and Melan-A specific CTL recognition of the non-natural Melan-A_{26-35} A27L analogues.*

Peptide name	Peptide sequence	HLA-A*0201 binding	CTL recognition
		Competitor activity [a] IC_{50} [nM]	Antigenic activity CE_{50} [nM]
Melan-A_{27-35}	AAGIGILTV	50	4
Melan-A_{26-35}	EAAGIGILTV	20	0.25
Melan-A_{26-35} A27L	ELAGIGILTV	2	0.01
Melan-A_{26-35} E26A/A27L	ALAGIGILTV	0.5	0.001
Flu MA	GILGFVFTL	< 0.01	-
Amino-protected analogs			
[Ac]-Melan-A	[Ac]-ELAGIGILTV	30	0.6
[NMeE1]-Melan-A	[NMeE]-LAGIGILTV	4	0.03
[pE1]-Melan-A	[Pyro]-ELAGIGILTV	1.8	0.002
[βA1]-Melan-A *	[βA]-LAGIGILTV	0.5	0.01
[βE1]-Melan-A	[βE]-LAGIGILTV	2	0.4
[βD1]-Melan-A	[βD]-LAGIGILTV	10	0.1
[NOH-G1]-Melan-A	[NOH]-GLAGIGILTV	0.6	0.01
[dE1]-Melan-A	[dE]-LAGIGILTV	3	0.6
[dL2]-Melan-A	E-[dL]-AGIGILTV	> 1000	4
[NMeL2]-Melan-A	E-[NMeL]-AGIGILTV	> 1000	30
[ψ_{1-2}(NH-CO)]-Melan-A	E-[ψ(NH-CO)]-LAGIGILTV	500	0.4
Carboxy-protected analogs			
[αMeL8]-Melan-A	ELAGIGI-[αMeL]-TV	4	p_1 0.01 p_2 3
[dL8]-Melan-A *	ELAGIGI-[dL]-TV	500	20
[amide]-Melan-A *	ELAGIGILTV-[$CONH_2$]	20	0.02

Table IB Cont'

Peptide name	Peptide sequence	HLA-A*0201 binding Competitor activity [a] IC_{50} [nM]	CTL recognition Antigenic activity CE_{50} [nM]
Double-protected analogs			
[NMeE1, amide]-Melan-A	[NMeE]-LAGIGITV-[$CONH_2$]	> 1000	1
[NMeE1, αMeL8]-Melan-A	[NMeE]-LAGIGI-[αMeL]-TV	p_1 110 p_2 >1000 [c]	p_1 0.5 p_2 20
[pE1, amide]-Melan-A	[Pyro]-ELAGIGILTV-[$CONH_2$]	200	0.3
[pE1, αMeL8]-Melan-A	[Pyro]-ELAGIGI-[αMeL]-TV	p_1 20 p_2 200	p_1 0.02 p_2 3
[βA1, amide]-Melan-A	[βA]-LAGIGILTV-[$CONH_2$]	28	0.03
[βA1, αMeL8]-Melan-A	[βA]-LAGIGI-[αMeL]-TV	p_1 3 p_2 70	p_1 0.02 p_2 0.06
[βE1, amide]-Melan-A	[βE]-LAGIGILTV-[$CONH_2$]	400	0.3
[βE1, αMeL8]-Melan-A	[βE]-LAGIGI-[αMeL]-TV	p_1 30 p_2 300	p_1 0.3 p_2 0.015
[βD1, amide]-Melan-A	[βD]-LAGIGILTV-[$CONH_2$]	500	10
[βD1, αMeL8]-Melan-A	[βD]-LAGIGI-[αMeL]-TV	p_1 100, p_2 300	p_1 0.2 p_2 0.02
[NOHG1, amide]-Melan-A	[NOH]-GLAGIGILTV-[$CONH_2$]	45	0.04
[NOHG1, αMeL8]-Melan-A	[NOH]-GLAGIGI-[αMeL]-TV	p_1 1 p_2 50	p_1 0.15 p_2 30

[a] Competitor activity was measured on the basis of the inhibition of recognition of the tyrosinase$_{368-376}$ (YMDGTMSQV, SEQ ID NO:46) antigenic peptide in the context of HLA-A*0201 by the tyrosinase peptide-specific CTL clone 156/34.

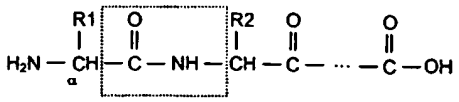
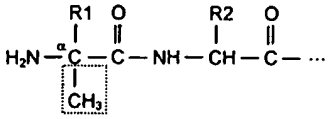
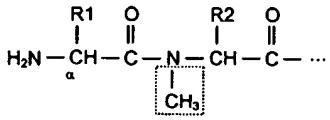
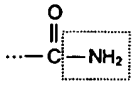
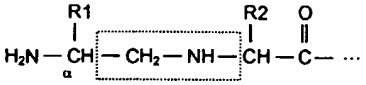
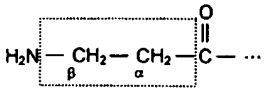
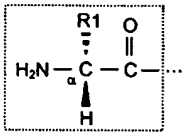
[b] In the case of α-methylated modified peptide, the two enantiomers separated by HPLC are indicated as p_1 and p_2 see "Material and Methods" peptide synthesis part for more details.

[c] Melan-A designation correspond to Melan-A$_{26-35}$ A27L.

* Modifications previously described in the initial patent.

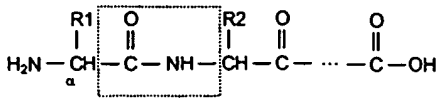
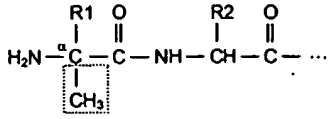
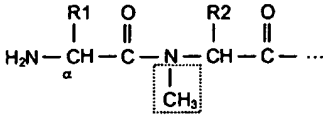
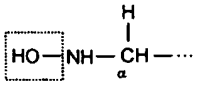
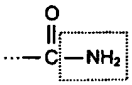
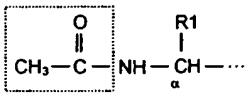
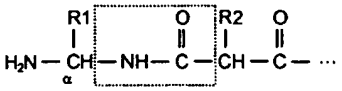
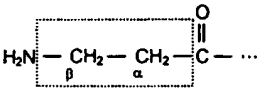
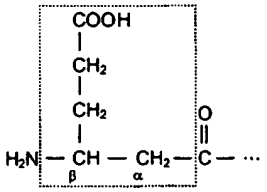
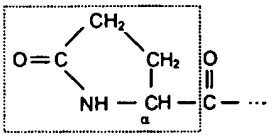
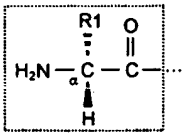
SEQ ID NOs (from top to bottom) = 1, 2, 3, 44, 45, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, and 43.

Table IIA : *Description, designation and structure of modifications of the MelanA$_{26-35}$A27L antigen*

Description	Designation	Stucture	Protection[a]
Peptide bond	-		-
Methylation of α carboxy	αMe-peptide		A, C
Methylation of nitrogen engaged in peptidic bond	NMe-peptide		C
Amidation terminal carboxylic function	Amide-peptide		C
Reduced bond	[Ψ_{1-2} (CH_2-NH)]		C
β amino-acid	βalanine βA		A
D-amino-acid	d-a.a.		C

[a] : A and C indicate efficient amino terminal and carboxy terminal protection respectively.

Table IIB : *Description, designation and structure of modifications of the MelanA$_{26-35}$A27L an*

Description	Designation	Stucture	Protection[a]
Peptide bond	-		-
Methylation of α carboxy	αMe-peptide		A, C
Methylation of nitrogen engaged in peptidic bond	NMe-peptide		A, C
Hydroxylation of terminal nitrogen	NOH-peptide		A
Amidation terminal carboxylic function	Amide-peptide		C
Acetylation of terminal nitrogen	Acetyl-peptide		A
Retro-inverso bond	[Ψ$_{1-2}$(CO-NH$_2$)]		A, C
β amino-acid	βalanine βA		A
β amino-acid	βglutamic acid βE		A
Cyclic amino-acid	Pyro-glutamic acid pE		A
D-amino-acid	d-a.a.		A, C

[a] : A and C indicate efficient amino terminal and carboxy terminal protection respectively.

EXAMPLE 2

Degradation of Melan-A_{26-35} A27L and analogs by peptidases in human serum.

For degradation studies, peptides were prepared in solution in 10 % DMSO at 10^{-3}M. Peptides were added to preheated (15 min at 37°C before the assay) human serum to a final concentration of 2.5 10^{-4} M and incubated at 37°C. At different times, an aliquot (100 µl) was removed and the enzymatic reaction stopped by addition of 11 µl trifluoroacetic acid (TFA). Precipitated serum proteins were pelleted by centrifugation at 15,000 rpm for 10 min at 4°C. The supernatants were frozen and preserved at -20°C until analysis. The following controls were performed : (1) Peptide recovery in supernatant after acidic precipitation is determined and titrated by HPLC analysis of a peptide solution (10^{-3}M) containing or not 10 % TFA. (2) Peptide stability in a 10 % TFA solution was checked. Under these conditions, Melan-A_{26-35} A27L and its substituted analogs were stable and did not precipitate in presence of 10 % TFA. (3) The HPLC profile of a sample of serum alone precipitated by TFA was recorded to detect peaks corresponding to non precipitated peptides present in the serum. (4) The absence of DMSO influence on serum proteases activity was checked.

The sequences of the degradation fragments were determined and quantified by on-line RP-HPLC/ESI-MS. The following HPLC conditions were used : - i) column : 4.6 mm x 25 cm, C18 (Beckman), - ii) gradient : 5 - 60 % CH_3CN (0.08 % TFA in H2O, 0.08 % TFA in CH3CN), 0-30 min, - iii) UV detection : 215 nm using a Waters detector, - iv) flow : 0.8 ml/min. The surface of peaks in Reconstructed Ion Current (RIC) analysis provides information of the quantity of the different degradation products. Finally, qualitative and quantitative information on the Melan-A_{26-35} A27L degradation products in human serum were obtained. Chromatographic conditions were the same as above except for the gradient. MS analysis was performed with a TSQ-700 Finnigan Mat mass spectrometer (San Jose, CA, USA). The entire HPLC flow was introduced into the ESI source. The capillary before the MS detection system was heated to 250°C. Nitrogen was used as a nebulizing gaz (sheath gaz flow : 80 psi, auxiliary gaz : 10 psi). The needle voltage was maintained at 5 kV. MS data (positive ions) were collected in the full scale mode (m/z 160 - 1400) at 2 ms per step.

The kinetic studies showed that Melan-A_{26-35} A27L was rapidly degraded (t1/2 = 40 min) by peptidases present in human serum. On line RP-HPLC/ESI-MS analysis

allowed us to identify and quantify the degradation products resulting from Melan-A_{26-35} A27L proteolysis. These experiments allowed us to define the exact pathway of Melan-A_{26-35} A27L degradation. The degradation process of Melan-A_{26-35} A27L follows two main mechanisms involving amino- and dipeptidyl-carboxy-peptidase (ACE : angiotensin-converting enzyme sub-family) activities and the two first peptide bonds sensitive to peptidases are Glu1-Leu2 and Leu8-Thr9. Different analogs with structural modifications at one (or both) peptidase-sensitive position(s) were synthesized and their degradation properties in serum were tested (Table IIIA). Resistance towards aminopeptidase was obtained by substitution of Leucine residue at position 2 (L2) by αMethyl-leucine residue or by substitution of glutamic acid residue at position 1 (E1) by βAlanine residue. In order to prevent degradation by dipeptidyl-carboxy-peptidase we replaced L8 by αMeL8, or T9 by NMeT9 or dT9. The 8-9 peptide bond was also modified with a reduced peptide bond ($\Psi(CH_2\text{-}NH)$) or realized amidation of the terminal carboxyl group to avoid carboxy-peptidase degradation. One-site protection was efficient to protect the peptide bond locally at the amino side or at the carboxy side of the peptide but did not result in peptides with significantly improved stability indicating that protection at both sites is needed to stabilize the peptide (data not shown). Indeed, all the two-sites protected analogs were rendered fully resistant (t1/2 >> 4 h) to peptidase activities. Further degradation studies have shown that the double protected analogs display a stability in human serum superior to 24h except for the [αMeL2, amide]-Melan-A_{26-35} A27L analog with an half-life about 12h (summarized Table IIIA). Five fully stabilized peptides were obtained:

[αMeL2, αMeL8]-Melan-A_{26-35} A27L (SEQ ID NO: 13)

[αMeL2, V10 amide]-Melan-A_{26-35} A27L (SEQ ID NO: 14)

[αMeL2, NMeT9]-Melan-A_{26-35} A27L (SEQ ID NO: 15)

[αMeL2, dT9]-Melan-A_{26-35} A27L (SEQ ID NO: 16)

[βA1, $\Psi_{8-9}(CH_2\text{-}NH)$]-Melan-$A_{26-35}$ A27L (SEQ ID NO: 17)

Table IIIA. *Stability in human serum of the modified Melan-A_{26-35} A27L analogues.*

Peptide name	Peptide sequence	Stability in human serum[a] Half-life $t_{1/2}$ (min)
Melan-A_{27-35}	AAGIGILTV	5
Melan-A_{26-35}	EAAGIGILTV	45
Melan-A_{26-35} A27L	ELAGIGILTV	40
[αMeL2, αMeL8]-Melan-A	E-[αMe]-LAGIGI-[αMeL]-TV	24 h
[αMeL2, V10-amide]-Melan-A	E-[αMe]-LAGIGILTV-[$CONH_2$]	12 h
[αMeL2, NMeT9]-Melan-A	E-[αMe]-LAGIGIL-[NMeT]-V	24 h
[αMeL2, dT9]-Melan-A	E-[αMe]-LAGIGIL-[dT]-V	24 h
[βA1, $\psi_{8-9}(CH_2\text{-}NH)$]-Melan-A	βALAGIGIL-[$\psi(CH_2\text{-}NH)$]-TV	24 h

[a] : The half-life of the peptide was calculated after incubation in human serum and HPLC-ESI-MS analysis.
Sequences from top to bottom: SEQ ID NO: 1, 2, 3, 13, 14, 15, 16 and 17

In order to further prevent proteolysis of Melan-A peptide, additional peptide analogs with different structural modifications at one (or both) peptidase-sensitive position(s) and tested their degradation properties. Resistance towards aminopeptidase was obtained by substitution of glutamic acid residue at position 1 (E1) by β̃amino acid residues (βE1, βA1 or βD1), by nitrogen-methylated residue (NMeE1) or by glycine residue bearing hydroxylation of the terminal nitrogen group (NOH-G1). Modification of second position (L2) was achieved by replacing by αMethyl-L2, NMethyl-L2 or dL2 residue. To prevent degradation by dipeptidyl-carboxy-peptidase we replaced L8 by αMeL8 or synthesized carboxy amidated peptide. Finally, peptide bond alterations like retro-inverso bond Ψ(NH-CO) were included to prevent amino-terminal degradation (Table IB and IIB).

To obtain fully protected analogs, the modifications of the peptides best HLA-A*0201 binding and Melan-A specific CTL recognition were combined (Table IA and described hereafter). All the two-site protected analogs were fully resistant (t1/2 >> 10h min or >> 24h) to peptidase activities (Table IIIB). All the amino acid modifications of the Melan-A antigen describe here and summarized in Table IB were efficient to locally protect the peptide against proteases degradation (Table IIIB). As described *supra*,

amidation protected efficiently against carboxypeptidase, but the half-life of such double protected amidated analog was less long (t1/2 ~12h) (Table IIIA) compared to other protections (i.e. αMe) with a half-life very superior to 24 hours (Table IIIA). Even in the case of the amidated peptides, the half-life of the double protected modified peptides was dramatically increased compared to Melan-A_{26-35} A27L peptide (t1/2 : 40min).

Table IIIB. *Stability in human serum of the non-natural Melan-A26-35 A27L analogues.*

Peptide name	Peptide sequence	Stability in human serum[a] Half-life $t_{1/2}$ (min)
Melan-A_{27-35}	AAGIGILTV	5
Melan-A_{26-35}	EAAGIGILTV	45
Melan-A_{26-35} A27L	ELAGIGILTV	40
[NMeE1, αMeL8]-Melan-A	[NMeE]-LAGIGI-[αMeL]-TV	24 h
[NMeE1, amide]-Melan-A	[NMeE]-LAGIGILTV-[$CONH_2$]	12 h
[pE1, amide]-Melan-A	[Pyro]-ELAGIGILTV-[$CONH_2$]	11 h
[pE1, αMeL8]-Melan-A	[Pyro]-ELAGIGI-[αMeL]-TV	24 h
[βA1, amide]-Melan-A	[βA]-LAGIGILTV-[$CONH_2$]	11 h
[βA1, αMeL8]-Melan-A	[βA]-LAGIGI-[αMeL]-TV	24 h
[βE1, amide]-Melan-A	[βE]-LAGIGILTV-[$CONH_2$]	20 h
[βE1, αMeL8]-Melan-A	[βE]-LAGIGI-[αMeL]-TV	24 h
[βD1, amide]-Melan-A	[βD]-LAGIGILTV-[$CONH_2$]	12 h
[βD1, αMeL8]-Melan-A	[βD]-LAGIGI-[αMeL]-TV	24 h
[NOHG1, amide]-Melan-A	[NOH]-GLAGIGILTV-[$CONH_2$]	13 h
[NOHG1, αMeL8]-Melan-A	[NOH]-GLAGIGI-[αMeL]-TV	24 h

[a] : The half-life of the peptide was calculated after incubation in human serum and HPLC-ESI-MS analysis. Sequences from top to bottom: SEQ ID NO: 1, 2, 3, 33, 32, 34, 35, 36, 37, 38, 39, 40, 41, 42 and 43.

EXAMPLE 3

HLA-A*0201 binding properties of Melan-A_{26-35} A27L and its analogs.

The peptide binding capacity to HLA-A*0201 was assessed in a functional competition assay based on inhibition of recognition of the antigenic peptide tyrosinase$_{368-376}$ (YMDGTMSQV, SEQ ID NO: 46) by the HLA-A*0201-restricted CTL clone 156/34. Various concentrations of competitor peptides (50 µl) were incubated with 51Cr-labeled T2 cells (50µl) (1000 cells/well) for 15 min at room temperature. A suboptimal dose (1 nM) of the antigenic peptide tyrosinase$_{368-376}$ (50 µl) was then added together with specific CTL (5000 cells/well) (50 µl). Chromium release was measured after 4h incubation at 37°C. The concentration of each competitor peptide required to achieve 50% inhibition of target cell lysis was then determined (IC50).

The ability of the structurally modified analogs to bind to HLA-A*0201 was assayed and compared to the parental peptide, Melan-A_{26-35} A27L. As shown in Table IA, all the modified resistant peptides display reduced affinity for HLA-A*0201 compared to the peptide Melan-A_{26-35} A27L. However, the decrease of HLA-A*0201 binding is not similar for all the analogs. The modified analog [αMeL2, NMeT9]-Melan-A_{26-35} A27L displays an important reduction of HLA-A*0201 binding (IC_{50} 500nM). In contrast the peptides [αMeL2, αMeL8]-Melan-A_{26-35} A27L (IC_{50} 9nM) or [βA1, $\Psi_{8-9}(CH_2\text{-}NH)$]-Melan-$A_{26-35}$ A27L (IC_{50} 20nM) have an HLA-A*0201 affinity relatively close to that of the Melan-A_{26-35} (IC_{50} 9nM) or Melan-A_{26-35} A27L peptide (IC_{50} 2nM).

Most of the structural modification of the Melan-A_{26-35} A27L antigenic peptide lead to a reduction ([Ac]-Melan-A IC_{50} 30nM, [Ψ_{1-2}(NH-CO)]-Melan-A IC_{50} 500nM, see examples) or a complete loss of peptide binding to HLA-A*0201 ([NMeL2]-Melan-A IC_{50} >1000nM, [dL8]-Melan-A IC_{50} 500nM) (Table 1B). However, some of the peptides of this invention having a single modification have a very similar HLA-A*0201 binding compared to Melan-A_{26-35} A27L (IC_{50} 2nM), ([βE]-Melan-A IC_{50} 2nM, [NOH-G1]-Melan-A IC_{50} 0.6nM, [αMeL8]-Melan-A IC_{50} 4nM) (Table IB).

In order to obtain fully protected peptide, the amino-acid modifications that afforded the greatest HLA-A*0201 binding affinity were combined and analogs generated

with amino acid modifications that have HLA-A*0201 binding properties that are very close to those observed for the parental Melan-A_{26-35} A27L peptide ([βA, αMeL8]-Melan-A p1 IC_{50} 3nM, [NOH-G1, αMeL8]-Melan-A IC_{50} 1nM, see Table IB).

EXAMPLE 4

Assessment of Antigen recognition by Melan-A_{26-35} A27L clone and polyclonal specific CTL.

Antigen recognition was assessed using chromium-release assays. Target cells (T2 cells) were labeled with ^{51}Cr for 1h at 37°C and washed three times. Labeled target cells (1000 cells in 50μl) were then added to various concentrations of antigenic peptide (50μl) in V-bottom 96-well plates for 15 min before the addition of the effector cells. A polyclonal CTL line specific for Melan-A_{26-35} A27L was used as effector cells and was then added (5000 cells in 100μl) at a defined effector to target cell ratio. Chromium release was measured in 100 μl of supernatant harvested after 4h of incubation at 37°C. The percentage of specific lysis was calculated as following: % specific lysis = [(experimental release – spontaneous release)/(total release – spontaneous release)] x 100.

The peptides (Table 1A) were tested for their capacity to sensitize target cells to lysis by Melan-A_{26-35} A27L specific CTL and compared with that of Melan-A_{27-35}, Melan-A_{26-35}, and Melan-A_{26-35} A27L. Figure 1 depicts the recognition of the Melan-A_{26-35} A27L modified analogs by specific CTL raised against Melan-A_{26-35} A27L. The recognition of non-natural Melan-A analogs by Melan-A specific CTL is evaluated by chromium release assay. The target cells use is the HLA-A2+ T2 cells and the ratio effector-target is 10/1. The CTL raised against Melan-A are derived from a polyclonal culture.

As shown in Figure 1 and summarized in Table IA, all the modified Melan-A_{26-35} A27L analogs were able to sensitize target cells to CTL lysis, but with different concentration ranges. In all the cases, the structural modifications of the peptides resulted in a decrease in the efficiency of Melan-A specific CTL recognition compared to that of the Melan-A_{26-35} A27L peptide. The modified analog [αMeL2, dT9]-Melan-A_{26-35} A27L is recognized as efficiently as the natural nonapeptide Melan-A_{27-35}. Two peptides, [βA1, $\Psi_{8-9}(CH_2\text{-}NH)$]-Melan-$A_{26-35}$ A27L and [αMeL2, NMeT9]-Melan-A_{26-35} A27L are able to trigger the lysis of target cells by Melan-A specific CTL in a range of concentration

comparable to that of the parental decapeptide Melan-A_{26-35}. The modified peptides [αMeL2, αMeL8]-Melan-A_{26-35} A27L and [αMeL2, amide]-Melan-A_{26-35} A27L are more efficiently recognized by Melan A_{26-35} A27L-specific CTL than the parental decapeptide Melan-A_{26-35}. Finally, the two peptide analogs [αMeL2, αMeL8]-Melan-A_{26-35} A27L and [αMeL2, amide]-Melan-A_{26-35} A27L that were best recognized by CTLs were selected for further immunogenicity experiments.

The recognition of additional modified Melan-A_{26-35} A27L analogs by specific CTLs was also assayed (summarized in Table IB). A description of the modifications is set forth in Table IIB. Most of the structurally modified peptides were able to sensitize target cells to lysis by Melan-A-specific CTL in nanomolar CE_{50} range. The CTL recognition of the modified Melan-A analogs correlate with the MHC binding data and poor HLA-A2 binder peptides are weakly recognized by CTL. Interestingly, some modified peptides were recognized as well as the Melan-A_{26-35} A27L peptide ([βA1, αMeL8]-Melan-A p2, [βD1, αMeL8]-Melan-A p2, and [βE1, αMeL8]-Melan-A p2, see Table IB).

EXAMPLE 5

Generation of CTLs specific for modified Melan-A_{26-35} A27L analogs.

Peripheral blood lymphocytes (PBL) from HLA-A*0201+ healthy donors were isolated by centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). CD8+ lymphocytes were isolated by positive selection magnetic cell sorting using a miniMACS device (Miltenyi Biotec GmBH, Sunnyvale, CA). The resulting population routinely contained >75% CD8+ T cells and were used as responder cell populations. Purified CD8+ T cells were plated at 1 x 10^6 cells/well together with 2 x 10^6 stimulator cells/well in 24-well plates in a total volume of 2 ml of Iscove's medium supplemented with 8 % of human serum, Asn, Arg, and Gln (complete medium) in the presence of IL-7 (10 ng/ml), IL-2 (10 U/ml) and 1μM of stimulating peptide . Stimulator cells were prepared as follows : 2 x 10^6 cells derived from CD8- population after miniMACS CD8+ lymphocyte isolation were irradiated (3000 rad) and adjusted to the appropriate volume before addition to the CD8+-enriched responder cell population.

On day 7, cells were restimulated with peptide-pulsed T2 cells. T2 cells were incubated for 2h at 37°C serum-free medium (X-VIVO 10; BioWhittaker) with the

appropriate stimulating peptide (1 μM) and human γ2 microglobulin (3μg/ml). Peptide pulsed T2 cells, were washed, irradiated (10 000rad) adjusted to appropriate volume of complete medium supplemented with IL-7 (10ng/ml), IL-2 (10U/ml) and added to the lymphocyte culture (2 x 10^5 cells/well). Subsequent restimulations were performed weekly with peptide pulsed T2 cells. CTL activity was first tested at the end of the first restimulation using ELIspot assay for IFNγ production by peptide reactive CTL.

The immunogenicity of the modified Melan-A analogs was addressed in experiments of *in vitro* stimulation of PBL from healthy donors. In these experiments, the ability of different peptide analogs to drive the activation and expansion of antigen-specific T lymphocyte precursors was assayed. Typically, purified $CD8^+$ T lymphocytes were stimulated twice and the magnitude of the *in vitro* response was measured by two procedures: (i) ELISPOT IFN-γ, which allows an estimation of the frequency of Melan-A-specific T cells able to produce IFN-γ upon short term restimulation, and (ii) flow cytometry analysis with tetramers, which enables direct enumeration of Melan-A antigen-specific T cells independent of their functional capabilities. Additionally, the antigen-specific T cells can be purified to homogeniety by tetramer guided cell sorting.

The cytolytic activity of these T cell populations was addressed using ^{51}Cr release assay. The reference peptide for the *in vitro* stimulation experiments was the peptide Melan-A_{26-35} A27L analog. This peptide was shown previously to possess a marked increased immunogenicity as compared to the non-modified Melan-A antigenic peptides Melan-A_{26-35} and Melan-A_{27-35} (Valmori et al., *J. Immunol.* 1998, 160 : 1750-1758, Men et al., *J. Immunol.* 1999, 162 : 3566-3573). Figure 2 depicts the immunogenicity of the Melan-A_{26-35} A27L modified analogs. CTL specific for the non-natural modified Melan-A analogs were obtain by in vitro stimulation of PBL of healthy donor as describe in the Examples. After 14 days, the resulting cultured lymphocytes were evaluated by ELIspot assay for the number of peptide-specific IFNγ-secreting lymphocytes. The CR1-A2 target cells used were pulsed with different antigenic peptide and used as stimulating cells. The Melan-A designation in Figure 2 corresponds to modified peptide of Melan-A_{26-35} A27L antigen.

As shown in Figure 2, stimulation in the absence of peptide did not induce a detectable increase in the number of Melan-A specific lymphocytes and no Melan-A

IFNγ-secreting cells can be detected. In contrast, the peptide analog Melan-A_{26-35} A27L was able to generate specific CTL that recognize efficiently the natural peptide Melan-A_{26-35}. Interestingly, the two modified peptides were also able to generate a measurable increase in the number of Melan-A specific lymphocytes after *in vitro* culture of PBLs. Stimulation with both [αMeL2, αMeL8]-Melan-A_{26-35} A27L and [αMeL2, amide]-Melan-A_{26-35} A27L induced lymphocytes that efficiently recognize both the natural peptide, Melan-A_{26-35}, and the parental analog peptide, Melan-A_{26-35} A27L, indicating efficient peptide cross-reactivity of the CTL induced with the modified analogs (Figure 2). Importantly, the CTL generated by stimulation with the modified Melan-A analogs were able to efficiently recognize the tumor cells Mel 290, which naturally express Melan-A (Figure 3).

Figure 3 depicts the recognition of tumor cells by CTL generated with the Melan-A_{26-35} A27L non-natural modified analogs. CTL specific for the non-natural modified Melan-A analogs were obtained by *in vitro* stimulation. Tumor cell recognition by CTL specific for the Melan-A_{26-35} A27L modified analogs was evaluated by ELIspot assay for IFNγ secreting lymphocytes. The stimulating cells used were the Melan-A^+, HLA-$A*0201^+$ tumor cell line Mel 290 or the CR1A2 (Melan-A^-, HLA-$A*0201^+$) cells as control.

To confirm and extend these results, the tetramer-Melan-A A27L$^-$ $CD8^+$ and the lymphocyte specific for Melan-A (tetramer-Melan-A A27L$^+$ $CD8^+$) populations were sorted and their ability to function as cytolytic effectors upon stimulation with the natural Melan-A antigen was tested in chromium release assays. CTL generated by stimulation with the Melan-A_{26-35} A27L peptide were able to lyse T2 cells pulsed with Melan-A_{26-35} but not the T2 target cells alone. Similar results were obtained with CTL generated by stimulation with the Melan-A modified analogs [αMeL2, αMeL8]-Melan-A_{26-35} A27L and [αMeL2, amide]-Melan-A_{26-35} A27L (Figure 4). These CTL were able to kill T2 cells pulsed with the natural peptide Melan-A_{26-35} with the same efficiency as CTL induced by stimulation with Melan-A_{26-35} A27L. In all cases, only the tetramer$^+$ $CD8^+$ populations were reactive with the peptide Melan-A_{26-35} suggesting the absence of Melan-A-specific CTL in the tetramer$^-$ $CD8^+$ lymphocyte population and therefore confirming the purity of the sorted cell populations.

Figure 4 depicts the recognition of Melan-A_{26-35} by the CTL generated with the Melan-A_{26-35} A27L modified analogs. The cytolytic activity of the CTL was tested in a ^{51}Cr release assay after tetramer guided cell sorting of the Melan-A specific CD8+ tetramer+ and CD8+ tetramer - lymphocyte populations. T2 target cells were pulsed 1μM of Melan-A_{26-35} peptide and mixed with lymphocytes with an effector to target cell ratio of 5/1. As control, the same experiments were done with T2 cells that were not pulsed with the Melan-A_{26-35} peptide.

Finally, as shown in Figure 5, the CTL generated by stimulation with the modified Melan-A analogs present the same capability as the Melan-A_{26-35} A27L specific CTL to recognize and lyse the tumor cells Me 290 that naturally presented the Melan-A antigen. In Figure 5 CTLs specific for the modified Melan-A analog were generated after *in vitro* stimulation of PBLs with appropriate peptide as describe in the Examples. The CTL populations CD8+ tetramer+ and CD8+ tetramer - were sorted after flow cytometry analysis. The ability of the CTL CD8+ tetramer+ to lyse the tumor cell Mel 290 was assayed using a ^{51}Cr release assay. The CTL were incubated with ^{51}Cr labeled tumor cells at a ratio effector to target of 5/1. The recognition of tumor cells by CTL generated with the Melan-A_{26-35} A27L modified analogs is depicted in Figure 5.

The structurally modified peptides [αMeL2, αMeL8]-Melan-A_{26-35} A27L, [αMeL2, amide]-Melan-A_{26-35} A27L, are fully resistant to peptidase activity and retain both the antigenicity and, most importantly, the immunogenicity of the parental tumor antigen Melan-A_{26-35} A27L. These peptides are potent stimulators of anti-melanoma CTL immune response and would be useful for inducing an immunological response to melanoma cells.

The immunogenicity of additional modified Melan-A peptide analogs were assayed by stimulating $CD8^+$ enriched cells isolated from PBL of three healthy donors with seven modified peptides. The same experiment was performed with the Melan-A_{26-35} A27L peptide as control. For the second stimulation, irradiated T2 cells pulsed with the appropriate peptides were used. The cells were assayed by flow cytometry 7 days after the second stimulation (MC2) for the presence of $CD8^+$ A2/MelanA $tetramer^+$ cells. In the case of cultures stimulated with the modified Melan-A analogs, A2/MelanA $tetramer^+$ cells were detected in the $CD8^+$ cells population indicating that the A2/Melan-A

tetramer was fully or partially cross reactive with the analog specific CTL, as shown in the Figure 6.

Figure 6 depicts the antigenic specificity and tumor recognition of CTL induced with the modified Melan-A_{26-35} A27L peptides. After *in vitro* stimulation of PBL from healthy donor HD224 with appropriate non-natural modified peptide analog, the tetramer+ CD8+ lymphocyte population was sorted on a FACSvantage cell sorter (exemplified in Figure 6A for MelanA_{26-35} A27L). The gate define for cell sorting of tetramer+ CD8+ population is also represented. Cell sorting of the tetramer+ CD8+ population and subsequent *in vitro* expansion of lymphocyte was realized for all the stimulation with the different analogs with the same conditions. *In vitro* stimulation with the peptide Melan-A_{26-35} A27L was performed in the same condition as control. Cytolytic CTL activity was measured after *in vitro* expansion of the different CTL populations (see *supra*). Cytolytic assay was performed with ^{51}Cr labeled tumor cells with an increasing effector to target cell ratio. The Melan-A analog used for *in vitro* stimulation is indicated before the two panels in Figure 6.

Fig 6 B1-B8, depicts Melan-A antigenic peptide recognition by the modified Melan-A analogs-specific CTL. A chromium release assay was performed with T2 cells as target cell pulsed with increasing concentration of peptide, the effector to target cell ratio was 10/1. Fig. 6 C1-C8, depicts tumor cell line recognition by the modified Melan-A analogs specific CTL. A cytolytic assay was performed with ^{51}Cr labeled tumor cells with an increasing effector to target cell ratio. The Melan-A analog used for *in vitro* stimulation is indicated in the figures.

Cross reactivity of the A2/Melan-A tetramer allows direct evaluation of the efficacy of the modified analogs to generate Melan-A specific CTLs from PBLs of healthy donors.

The percentage of Melan-A specific cells obtained after stimulation with peptide Melan-A_{26-35} A27L differed from one donor to another and related to the frequency of Melan-A reactive precursors in the initial $CD8^+$ population (Table IV, HD224 : 2.8%, HD410 2.5%, HD220 0.3%). For the doubly protected modified analogs, the percentages of Melan-A specific cells were lower, similar or in some case higher than the percentage obtained with the parental peptide Melan-A_{26-35} A27L (Table IV). Interestingly, three

analogs ([βA1, αMeL8]-, [βE1, αMeL8]-, and [βD, αMeL8]-Melan-A_{26-35} A27L) were able to induce twice the percentage of Melan-A specific cells that were induced by the parental peptide Melan-A_{26-35} A27L in the cultures derived from all the healthy donors (see Table IV). These three modified peptides appear to be more immunogenic than the parental peptide in the *in vitro* assays.

Table IV. In vitro immunogenicity of the modified Melan-A_{26-35} A27L analogues.

Peptide used for stimulation	*In vitro* immunogenicity [a] (% of A2/ELA- tetramer$^+$ CD8$^+$ cells in the culture) HD224 [b]	HD410	HD220
No peptide	0.1	0.1	0.09
Melan-A_{26-35} A27L	2.8	2.5	0.3
[βA1, amide]-Melan-A	2.7	2.2	0.3
[βA1, αMeL8]-Melan-A p2	6.0	4.3	0.2
[βE1, αMeL8]-Melan-A p2	5.3	4.5	0.6
[βD1, αMeL8]-Melan-A p2	5.6	4.3	0.6
[NOHG1, amide]-Melan-A	3.1	2.0	0.3
[NOHG1, αMeL8]-Melan-A p1	2.0	1.0	0.1
[pE1, αMeL8]-Melan-A p1	1.0	0.5	0.2

[a] : Melan-A specific cells (A2/ELA- tetramer$^+$ CD8$^+$) were directly detected 7 days after the second stimulation (MC2) by flow cytometry by co-staining of the culture with A2/ELAtetramers^{PE} and CD8^{FITC} mAb (see Examples for more details).

[b] : HD : Healthy Donor.

Sequences from top to bottom: SEQ ID NO: SEQ ID NO: 3, 36, 37, 39, 41, 42 and 43.

EXAMPLE 6

Flow Cytometric immunofluorescence analysis and Melan-A tetramer guided cells sorting of specific CD8+ T lymphocytes.

The specificity of recognition of the lymphocytes was monitored by flow cytometry analysis after Melan-A_{26-35} A27L HLA-A*0201-tetramer binding. The use of Melan-A_{26-35} A27L-containing HLA-A*0201 tetramers for visualization and isolation of Melan-A-specific CTL has been described previously (Romero et al., *J. Exp. Med.*, 188(9):1641 (1998); Pittet et al., *J. Exp. Med.*, 190(5):705 (1999) incorporated herein by reference). Briefly, cells were incubated with tetramers (200 ng/sample) in 20 µl of PBS/2% FCS for 20 min at room temperature, then 20 µl of anti-CD8FITC mAb (Becton Dickinson, Basel, Switzerland) were added, and cells were incubated for an additional 30 min at 4°C. Cells were washed once in the same buffer and analyzed by flow cytometry using a FACScan (Becton Dickinson). Data analysis was performed using CellQuest

software (Becton Dickinson). The CD8+ tetramer+ and CD8+ tetramer- lymphocyte populations were sorted using a FACSVantage (Becton Dickinson) cell sorter.

After *in vitro* expansion, without specific stimulation, the antigenic specificity of the sorted cells was addressed with chromium release cytolytic assay. As demonstrated in Figure 6 B, the CTLs specific for the modified Melan-A analogs were able to recognize and lyse efficiently T2 target cell presenting the Melan-A_{26-35} A27L peptide, and also the Melan-A_{26-35} and Melan-A_{27-35} peptides. The Na8 cell line, which does not express Melan-A gene, was not susceptible to lysis by the CTLs specific for the modified Melan-A peptides indicating an absence of non-specific lytic activity (Figure 6C). The Na8 cells were efficiently lysed when pulsed with the Melan-A_{26-35} peptide. Importantly, the tumor cells line Mel 290 and Mel 275, which naturally express the Melan-A antigen on the cell surface, was also recognized by CTLs specific for modified Melan-A analogs. Thus, the CTLs induced with the modified Melan A_{26-36} A27L analogs displayed similar cytolytic specificity as the CTLs specific for the parental peptide Melan-A_{26-35} A27L and are fully capable of recognizing cells presenting complexes of the parental Melan-A peptides and lysing tumor cell lines which naturally express the Melan-A antigen.

EXAMPLE 7

IFN-γ ELIspot assay.

Nitrocellulose-lined microtiter plates (Multiscreen-HA, Millipore) were coated overnight with antibody to human IFN-γ (Mabtech) and washed six times. Lymphocytes (5×10^4 per well) were mixed with an equal number of target cells CR1-A2 alone or pulsed with peptide in Iscove's medium (200 µl) supplemented with 8% human serum and incubated 20 h at 37°C. After extensive washing with washing buffer (PBS, 01%-Tween 20), plates were incubated with biotinylated secondary antibody, then washed again and incubated with avidin conjugated with alkaline phosphatase. Then the plates were developed with BCIP substrate and the blue spots corresponding to the number of cells secreting IFN-γ were counted using Bioreader 2000 (Biosys, Germany)

In conclusion, disclosed herein are new structural modifications that provide modified Melan-A_{26-35} A27L analogs that are fully resistant to proteases degradation but having HLA-A*0201 binding and Melan-A-specific CTL recognition properties very similar to those of the Melan-A_{26-35} A27L parental peptide. These modified Melan-A analogs were able to induced Melan-A specific anti-tumor CTLs. Among these peptides, the analogs [βA1, αMeL8]-Melan-A, [βE1, αMeL8]-Melan-A and [βD1, αMeL8]-Melan-A were able to induce an increased number of Melan-A specific T cells compared to the parental Melan-A_{26-35} A27L peptide. These peptides which are more immunogenic than Melan-A are candidates for peptide immunotherpy of melanoma. For example, the modified peptides may be used to stimulate CTLs in vitro which recognize melanoma cells expressing the natural peptide and these CTLs could be introduced into a patient in need thereof to lyse the melanoma cells. Alternatively the modified peptides may be introduced into a subject in need thereof to stimulate the subject's own CTLs to mount a response against the cells expressing the natural peptide in complex with an MHC molecule.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

WE CLAIM:

1. Isolated peptide which binds to an HLA-A*0201 molecule wherein said peptide is a modified analog of Melan-A_{26-35} A27L (SEQ ID NO: 3) comprising

   (a) at least one modified amino acid at position 1 or 2, or

   (b) at least one modified amino acid at position 8, 9 or 10,

   wherein said modified analog of Melan A_{26-35} A27L has a half-life in human serum of at least about 12 hours.

2. The isolated peptide of claim 1 wherein the modified amino acid at position 1 or 2 is selected from the group consisting of a β-amino acid (β-aa), a D-amino acid (d-aa), an amino acid having a methylation of an α-carboxy (αMe-aa), an amino acid having a methylation of a nitrogen engaged in peptidic bond formation (NMe-aa) and an amino acid having a reduced bond ($\Psi_{1-2}(CH_2\text{-}NH)$.

3. The isolated peptide of claim 1 wherein the amino acid at position 1 or 2 is modified, wherein said modified amino acid is selected from the group consisting of an amino acid having a methylation of an α-carboxy (αMe-aa) and a β-amino acid (β-aa).

4. The isolated peptide of claim 3 wherein the amino acid having a methylation of an α-carboxy is a αMe-Glutamine.

5. The isolated peptide of claim 3 wherein the β-amino acid is a β-Alanine

6. The isolated peptide of claim 1 wherein an amino acid in position 8, 9 or 10 is modified, wherein said modified amino acid is a β-amino acid (β-aa), a D-amino acid (d-aa), an amino acid having a methylation of an α-carboxy (αMe-aa), an amino acid having a methylation of a nitrogen engaged in peptidic bond formation (NMe-aa) and an amino acid having a reduced bond ($\Psi_{1-2}(CH_2\text{-}NH)$).

7. The isolated peptide of claim 6 wherein the modified amino acid is an αMe-Leucine at position 8, an amidated valine at position 10, an NMe T at position 9, or a d-Thr at position 9.

8. The isolated peptide of claim 6 wherein the modified peptide comprises a reduced bond ($\Psi_{1-2}(CH_2\text{-}NH)$ between amino acids at positions 8 and 9.

9. The isolated peptide of claim 1, wherein said isolated peptide has a half life of at least about 24 hours.

10. The isolated peptide of claim 1 wherein said peptide has an amino acid sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43.

13. A composition comprising the isolated peptide of claim 9 and a pharmaceutically acceptable carrier.

14. The composition of claim 13 wherein the isolated peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43.

15. A method for stimulating proliferation of cytolytic T cells, comprising contacting a sample containing cytolytic T cell precursors with a complex of HLA-A*0201 and the isolated peptide of claim 1 in an amount sufficient to provoke proliferation of any cytolytic T cell precursors specific to said complex.

16. The method of claim 15 wherein the isolated peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43.

17. A polytope comprising one or more of the isolated peptides of claim 1 linked to another peptide.

18. The polytope of claim 17 wherein said isolated peptide is selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42 and SEQ ID NO: 43.

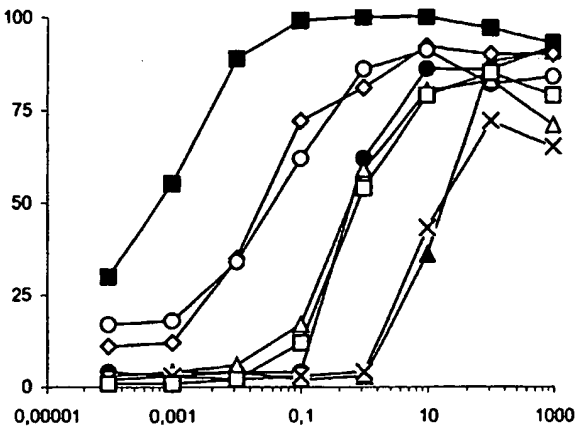


FIGURE 1

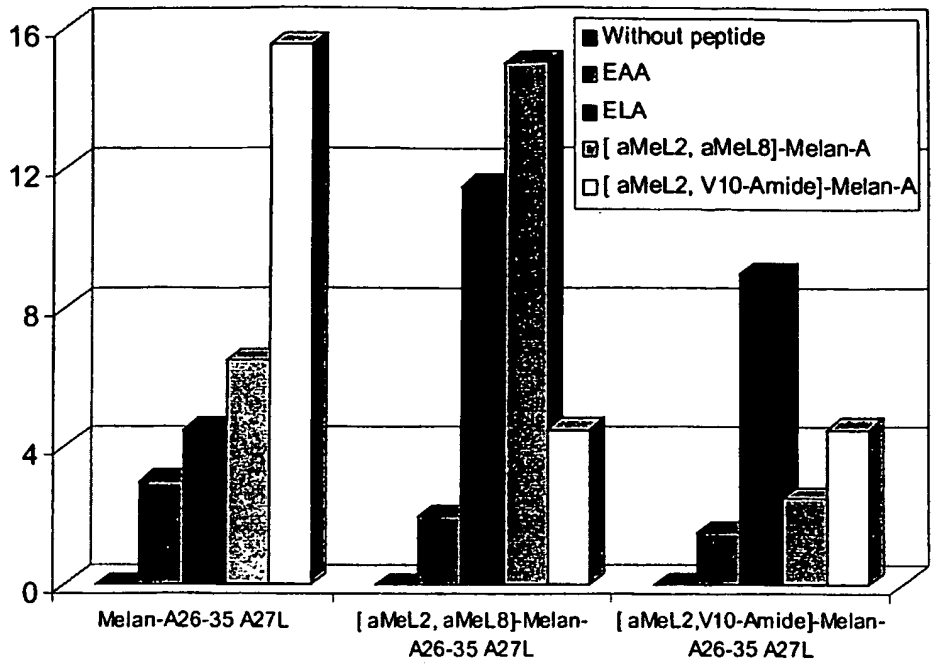

16
12
8
4
0
Without peptide
EAA
ELA
[aMeL2, aMeL8]-Melan-A
[aMeL2, V10-Amide]-Melan-A
Melan-A26-35 A27L
[aMeL2, aMeL8]-Melan-A26-35 A27L
[aMeL2,V10-Amide]-Melan-A26-35 A27L
Peptide use for PBL stimulation

FIGURE 2

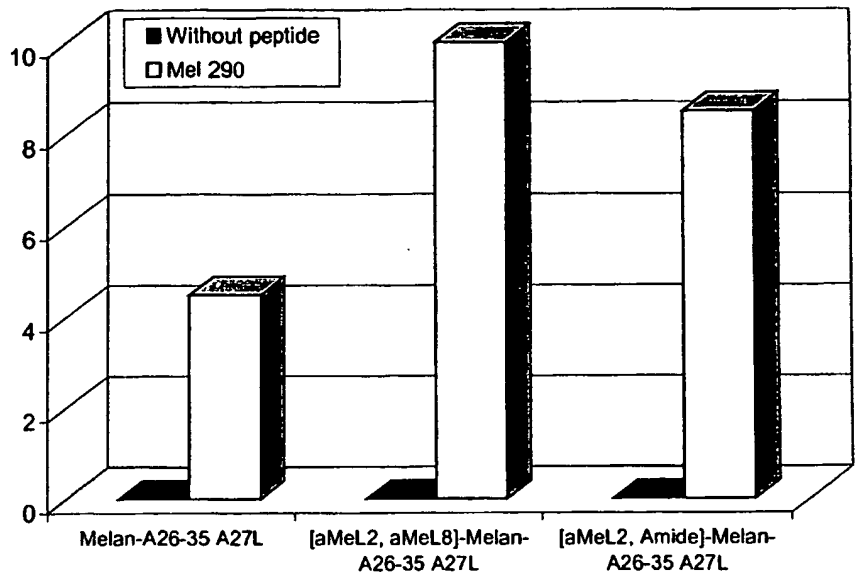
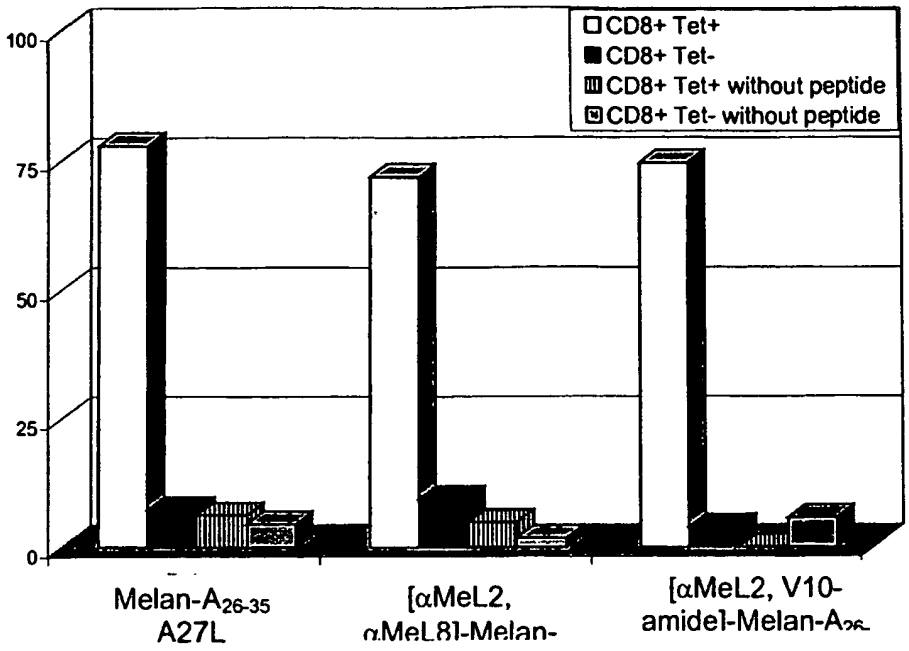

Without peptide
Mel 290
10
8
6
4
2
0
Melan-A26-35 A27L
[aMeL2, aMeL8]-Melan-A26-35 A27L
[aMeL2, Amide]-Melan-A26-35 A27L
Peptide use for PBL stimulation

FIGURE 3



Peptide use for PBL stimulation

FIGURE 4

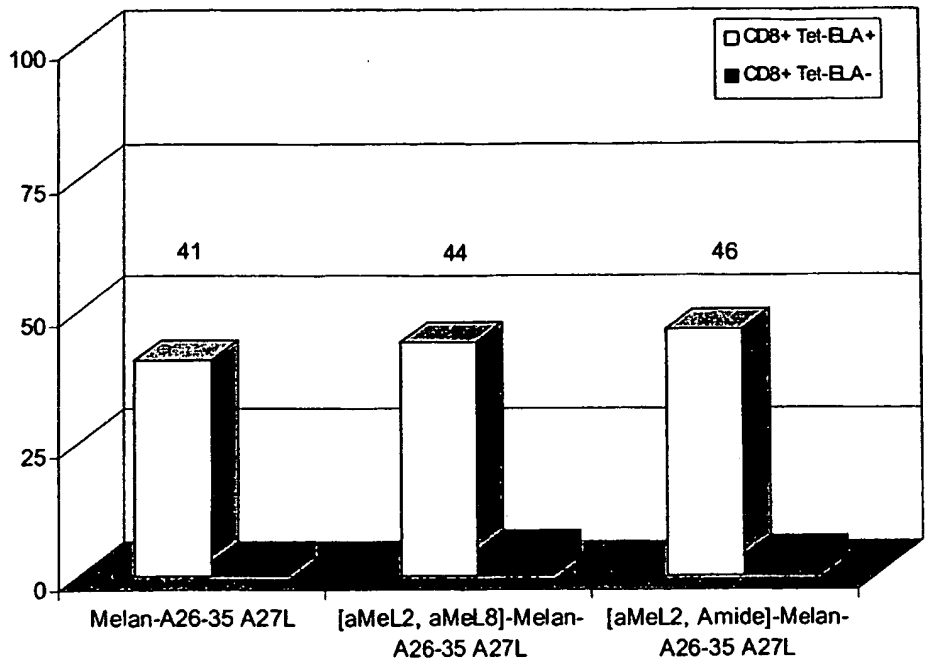

CD8+ Tet-ELA+
CD8+ Tet-ELA-
100
75
50
25
0
41
44
46
Melan-A26-35 A27L
[aMeL2, aMeL8]-Melan-A26-35 A27L
[aMeL2, Amide]-Melan-A26-35 A27L
Peptide use for PBL

FIGURE 5

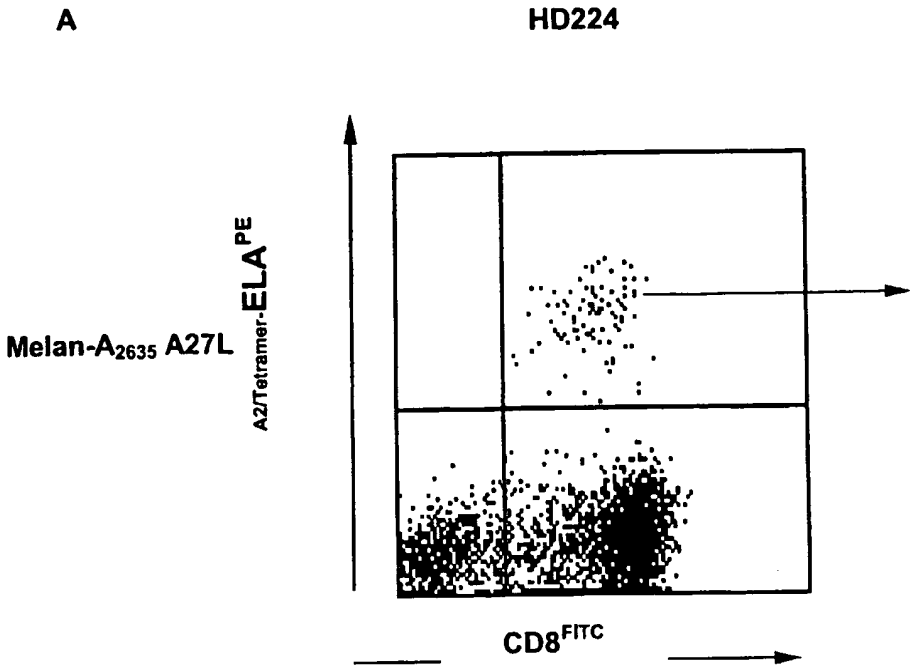
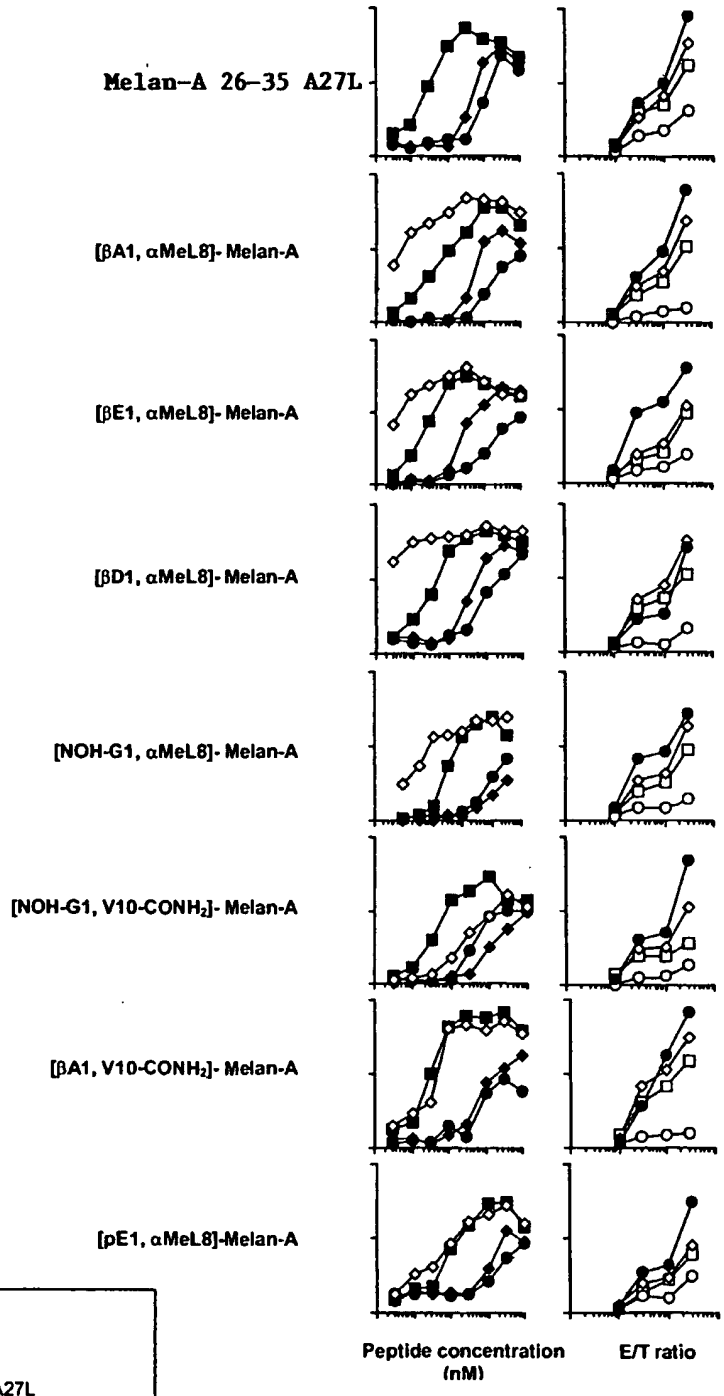


FIGURE 6A



Legend to figure

B : ● Melan-A_{26-35}
◆ Melan-A_{27-35}
■ Melan-A_{26-35} A27L
◇ Melan-A_{26-35} A27L analogue *

C : ● Na8 + Melan-A_{26-35}
○ Na8
◇ Mel 275
□ Mel 290

FIGURE 6B and 6C

* Melan-A_{26-35} A27L analogue corresponds to the analogue used for *in vitro* stimulation

LUD 5725 PCT.ST25.txt
SEQUENCE LISTING

<110> LUDWIG INSTITUTE OF CANCER RESEARCH

<120> Structurally Modified Peptides and Uses Thereof

<130> LUD 5725 PCT

<140> Not yet assigned
<141> 2002-05-14

<150> 60/290,646
<151> 2001-05-15

<160> 46

<170> PatentIn version 3.1

<210> 1
<211> 9
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a decamer peptide corresponding to residues 27-35 of Mela
n-A.

<400> 1

Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5

<210> 2
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a decamer peptide of Melan-A residues 26-35.

<400> 2

Gln Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 3
<211> 10
<212> PRT
<213> Artificial sequence

<220>

<223> This is a decamer peptide based on Melan-A residues 26-35.

<400> 3

Glu Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 4
<211> 9
<212> PRT
<213> artificial sequence

<220>
<223> This is a nonamer peptide based on Flu MA.

<400> 4

Gly Ile Leu Gly Phe Val Phe Thr Leu
1 5

<210> 5
<211> 10
<212> PRT
<213> artificial sequence

<220>
<223> This is a modified decamer peptide of Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The alanine at position 1 is a beta-alanine

<400> 5

Ala Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 6
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE

<222> (2)..(2)
<223> The amino acid residue at position 2 is an N-methyl-leucine.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The amino acid at position 2 is an N-methyl-leucine.

<400> 6

Glu Xaa Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 7
<211> 10
<212> PRT
<213> artificial sequence

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(2)
<223> The bond between amino acids at positions 1 and 2 is a reduced peptide bond.

<400> 7

Glu Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 8
<211> 10
<212> PRT
<213> Artificial sequence

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alpha-methyl-leucine.

<400> 8

Glu Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 9
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (9)..(9)
<223> The amino acid residue at position 9 is an N-Methyl-Threonine.

<400> 9

Glu Leu Ala Gly Ile Gly Ile Leu Xaa Val
1 5 10

<210> 10
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (9)..(9)
<223> The amino acid residue at position 9 is a D-Threonine.

<400> 10

Glu Leu Ala Gly Ile Gly Ile Leu Xaa Val
1 5 10

<210> 11
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The valine at position 10 contains an amidated valine.

<400> 11

Glu Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 12
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (8)..(9)
<223> The bond between residues at positions 8 and 9 is a reduced peptide bond.

<400> 12

Glu Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 13
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The amino acid at position 2 is an alpha-methyl-leucine.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alpha-methyl-leucine.

<400> 13

Glu Xaa Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 14
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The amino acid residue at position 2 is an alpha-methyl-leucine.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid residue at position 10 is an amidated valine.

<400> 14

Glu Xaa Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 15
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The amino acid residue at position 2 is an alpha-methyl-leucine.

<220>
<221> MISC_FEATURE
<222> (9)..(9)
<223> The amino acid residue at position 9 is an alpha-methyl-threonine
.

<400> 15

Glu Xaa Ala Gly Ile Gly Ile Leu Xaa Val
1 5 10

<210> 16
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-3
5.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The residue at position 2 is an alpha-methyl-leucine.

<220>
<221> MISC_FEATURE
<222> (9)..(9)
<223> The residue at position 9 is an N-methyl-threonine.

<400> 16

Glu Xaa Ala Gly Ile Gly Ile Leu Xaa Val
1 5 10

<210> 17
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-3
5.

<220>
<221> MISC_FEATURE
<222> (1)..(1)

<223> The amino acid at position 1 is a beta-alanine.

<400> 17

Ala Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 18
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is an acylated glutamic acid.

<400> 18

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 19
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is an N-methyl-glutamic acid.

<400> 19

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 20
<211> 10

<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> THis is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a pyro-glutamic acid.

<400> 20

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 21
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> THis is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a beta-alanine.

<400> 21

Ala Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 22
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> THis is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a beta-glutamic acid.

<400> 22

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 23
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> THis is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is D-glutamic acid.

<400> 23

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 24
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> THis is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a hydroxylated glycine wherein the terminal nitrogen is hydroxylated.

<400> 24

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 25
<211> 10

<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a D-glutamic acid.

<400> 25

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 26
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The amino acid at position 2 is a D-leucine.

<400> 26

Glu Xaa Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 27
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> The amino acid at position 2 is an N-methyl-leucine.

LUD 5725 PCT.ST25.txt

<400> 27

Glu Xaa Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 28
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(2)
<223> The peptide bond between residues 1 and 2 is a retro inverso peptide bond.

<400> 28

Glu Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 29
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alph-methyl-leucine.

<400> 29

Glu Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 30
<211> 10

<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is a D-leucine.

<400> 30

Glu Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 31
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan-A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid at position 10 is an amidated valine.

<400> 31

Glu Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 32
<211> 9
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified nonamer peptide based on Melan-A residues 27-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is an N-methyl-glutamic acid.

<220>
<221> MISC_FEATURE
<222> (9)..(9)
<223> The amino acid at position 9 is a amidated glutamic acid.

<400> 32

Xaa Leu Ala Gly Ile Gly Ile Thr Xaa
1 5

<210> 33
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is an N-methyl-lglutamic acid.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an N-methyl-leucine.

<400> 33

Xaa Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 34
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)

<223> The amino acid at position 1 is pyro-glutamic acid.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid at position 10 is an amidated valine.

<400> 34

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 35
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a pyro-glutamic acid

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alpha-methyl-leucine.

<400> 35

Xaa Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 36
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE

<222> (1)..(1)
<223> The amino acid at position 1 is a beta-alanine.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid at position 10 is an amidated valine.

<400> 36

Ala Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 37
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is an beta-alanine.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alpha-methyl-leucine.

<400> 37

Ala Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 38
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>

<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a beta-glutamic acid.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid at position 10 is an amidated valine.

<400> 38

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 39
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a beta-glutamic acid.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alpha-methyl-leucine

<400> 39

Xaa Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 40
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a beta-aspartic acid.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid at position 10 is an amidated valine.

<400> 40

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 41
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid position 1 is a D-aspartic acid.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid position 8 is an alpha-methyl-leucine.

<400> 41

Xaa Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 42
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-35.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a hydroxylated glycine.

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> The amino acid at position 10 is an amidated valine.

<400> 42

Xaa Leu Ala Gly Ile Gly Ile Leu Thr Xaa
1 5 10

<210> 43
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This is a modified decamer peptide based on Melan A residues 26-3
5.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> The amino acid at position 1 is a hydroxylated glutamic acid wher
ein the terminal nitrogen is hydroxylated.

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> The amino acid at position 8 is an alpha-methly-leucine.

<400> 43

Xaa Leu Ala Gly Ile Gly Ile Xaa Thr Val
1 5 10

<210> 44
<211> 10
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>

<223> This sequence is a decamer peptide based on the polypeptide Melan -A residues 26-35 with substitutions at positions 26 and 27.

<400> 44

Ala Leu Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

<210> 45
<211> 9
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This sequence is a nonamer peptide of Flu MA.

<400> 45

Gly Ile Leu Gly Phe Val Phe Thr Leu
1 5

<210> 46
<211> 9
<212> PRT
<213> ARTIFICIAL SEQUENCE

<220>
<223> This sequence is a nonamer of the tyrosinase peptide residues 368 -376.

<400> 46

Tyr Met Asp Gly Thr Met Ser Gln Val
1 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/15217

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/04, 38/08; C07K 4/12, 7/02
US CL : 530/323,300,332; 514/12; 424/277.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/323,300,332; 514/12; 424/277.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VALMORI et al. Enhanced Generation of Specific Tumor-Reactive CTL In Vitro by Selected Melan-A/MART-1 Immunodominant Peptide Analogues. The Journal of Immunology. 1998, Vol. 160, pages 1750-1758, see entire document.	1-10, 13-18
Y	US 6,218,363 B1 (BASERGA et al.) 17 April 2001, see entire document.	1-10, 13-18
Y	US 5,662,907 A (KUBO et al.) 02 September 1997, see entire document.	1-10, 13-18
Y	US 5,736,142 A (SETTE et al.) 07 April 1998, see entire document.	1-10, 13-18
Y	US 5,932,224 A (CHISARI et al.) 03 August 1999, see entire document.	1-10, 13-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 JUNE 2002	Date of mailing of the international search report 16 SEP 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Janice Ford for RON SCHWADRON, Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/15217

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST 2.1, MEDICINE/BIOTECH compendium databases on DIALOG), search terms: inventor names, melan a26-35 A27L, d-amino, b-amino, methylation, half life, increas?, b-alanine, amidated, alpha carboxy, reduced bond,